Prevalence of possible immune resistance mechanisms of acute leukemias within the context of vaccination strategies using the Wilms tumor gene-1 (*WT1*)

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- Nothing in biology makes sense except in the light of evolution. -

Theodisius Dobzhansky

Genetics and the Origin of Species
(1937, Columbia Univ. Press, New York)

Zusammenfassung

Das Wilms-Tumorgen 1 (WT1) repräsentiert eine der erfolgversprechendsten Zielstrukturen für spezifische immuntherapeutische Ansätze bei myeloischen Leukämien sowie bei einer Reihe weiterer hämatologischer und solider Neoplasien. Mehrere Studien, darunter eine Studie aus unserer Klinik zur klinischen Wirksamkeit und Relevanz von WT1 in Kombination mit KLH (engl. *keyhole limpet hemocyanin*) und hGM-CSF (engl. *Granulocyte-macrophage colony-stimulating factor*), zeigen eine hohe Immunogenität von WT1-Peptiden durch Induktion spezifischer T-Zellen bei 50-70% eigentlich austherapierter Patienten und Hinweise für klinische Effizienz [1-3].

Die allerdings nur selten dauerhaft etablierbaren T-Zell-Frequenzen stellen eine der wichtigsten Hürden auf dem Weg zur allgemeinen klinischen Anwendung dar. Bislang fehlen jedoch systematische Untersuchungen, die analysieren, aus welchem Grund die Konstitution eines immunologischen Gedächtnisses in diesem Kontext so herausfordend ist. Diese Arbeit sollte deshalb einen Einblick über die T-Zell-Funktionalität, die phänotypische Zusammensetzung der T-Zell-Population als auch über die lytische Effizienz WT1-spezifischer, zytotoxischer T-Zellen gegenüber leukämischen Blasten bieten (Abbildung 1).

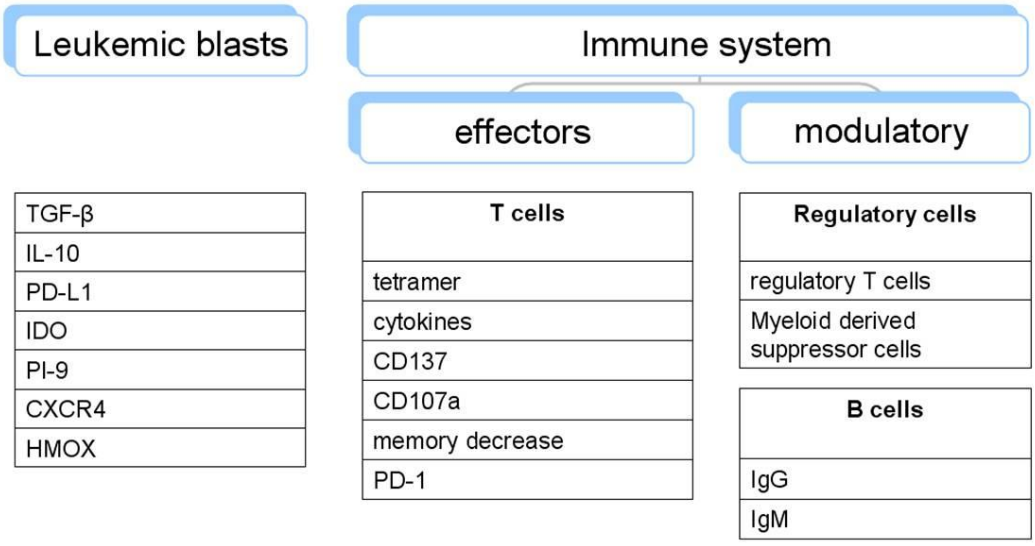

Abbildung 1: Übersicht über die in dieser Arbeit untersuchten Mechanismen, welche potentiell zu einer Einschränkung einer effektiven anti-Tumor-Immunantwort führen können.

Ergänzend zu den funktionellen Untersuchungen fehlen detaillierte Analysen über die Präsenz und Induktion verschiedener Blasten-Charakteristika, die indirekt die Sensitivität gegenüber der T-Zell-vermittelten Zytotoxizität vermindern, als auch

ausreichende Erkenntnisse über die direkte Suppression WT1-spezifischer T-Zellen durch die Leukämie.

Aus diesem Grunde sollte untersucht werden, ob und wie stark die Sensitivität von WT1-spezifischen T-Zellen gegenüber leukämischen Blasten von den Expressionsniveaus anti-zytotoxischer/apoptotischer Proteine und der Präsenz weiterer immunsupprimierender Faktoren abhängt. Hierunter fällt, neben der Sekretion von löslichen als auch der Präsentation von membranständigen Immunmodulatoren durch die Blasten, vornehmlich die Präsenz neu-induzierter immunregulatorischer Zellen.

Humorale Immunität gegenüber WT1 und KLH in AML-Patienten durch die Vakzinierung mit einem WT1-Epitop, KLH und GM-CSF

Das grundlegende Prinzip jeder Impfung ist in der Regel die Etablierung eines immunologischen Gedächtnisses gegen das Zielpathogen [4]. Idealerweise führt eine Impfung deshalb zu einer Aktivierung des kompletten Repertoires des Immunsystems. Im Falle der Einzelpeptid-basierten Immuntherapie werden durch das Antigen in den meisten Fällen nur die zytolytischen Effektorzellen, die ZTLs, aktiviert. Zur Unterstützung des Aktivierungsprozesses werden dem Vakzin deshalb Adjuvantien wie Monensin, KLH und hGM-CSF hinzugefügt, um eine Antigen-unspezifische Helferantwort von Tumorzellen zu induzieren. Dies sollte zur Freisetzung von zusätzlichem „natürlichem“ antigenen Material führen, welches eine Reaktion des kompletten Immunsystems hervorrufen sollte. Untersuchungen, die die humorale Antwort der Patienten gegenüber dem WT1-Protein evaluierten, zeigten, dass im Zuge der Vakzinierung eine Induktion von WT1-spezifischen Immunglobulinen stattfindet. Diese Induktion beschränkt sich allerdings nur auf den Isotyp M (IgM), der von naïven B-Zellen produziert wird und als Marker für eine Initialantwort dienen kann. Der sogenannte *isotype-switch* (Isotypen-Klassenwechsel), die Produktion des Isotyps G (IgG) an Stelle von IgM, welcher die Etablierung eines immunologischen B-Zell-Gedächtnisses repräsentiert, findet in unserem Patientenkollektiv im Laufe der Vakzinierung allerdings nicht statt. Dies kann vermutlich auf eine nicht ausreichende Stimulation von CD4-Helfer-Zellen durch das MHC-Klasse I-restringierte WT1-Peptid zurückgeführt werden, eine Annahme, die durch den nachweisbaren Isotypen-Klassenwechsel bei KLH-und hGM-CSF-spezifischen Immunglobulinen gestützt wird (Absatz 4.1).

Phänotypische und funktionale Analysen der zellulären Immunität in AML-Patienten vakziniert mit einem WT1-Epitop, KLH und GM-CSF

Weitere Untersuchungen sollten Aussagen zur T-zellulären Funktionalität als auch zur Zusammensetzung der T-Zell-Population im Vakzinierungsverlauf zulassen. Die Bestimmung erfolgte zu zwei Zeitpunkten, dem Vakzinierungsstartpunkt und während des Vakzinierungsverlaufs in Woche 10.

Die Ergebnisse dieser Experimentenreihe zeigten zwar ein heterogenes Bild, was die Zytokinfreisetzung und/oder Zytolysekapazität betrifft, wiesen aber keine signifikante Korrelation zum klinischen Ergebnis auf. Allerdings konnte bei diesen Messungen nachgewiesen werden, dass eine bereits bestehende oder während der Therapie akquirierte T-Zell-Anergie den klinischenVerlauf stark beeinflusst.

Der wiederholte Kontakt einer T-Zelle mit ihrem Antigen führt zur gesteigerten Expression von Faktoren, die die Aktivierungsschwelle der T-Zelle bei einem neuen Kontakt erhöhen [5, 6]. Dies führt letztendlich zu einem anergischen Status der Zelle, welcher durch eine geringe bis gar nicht vorhandene Reaktivität der T-Zelle gekennzeichnet ist. Dieser Prozess dient eigentlich dazu, nach der Pathogen-Eradikation die Immunantwort langsam wieder einzudämmen [7], ist im Kontext der *Immune-Surveillance* allerdings kontraproduktiv.

In dieser Arbeit konnte gezeigt werden, dass sich die Expression des Immun-Regulators PD-1 (*programmed death-1 receptor*) negativ auf die mittlere Überlebensdauer der betroffenen Patienten auswirkt (p=0,05). Ein ähnliches Bild zeigte sich für diesen Faktor auch in Bezug auf die biologische Aktivität des Vakzins, welche über die Abnahme von WT1-RNA-Transkripten zu denselben Zeitpunkten bestimmt wurde. Patienten mit geringem PD-1-Niveau zeigen eine deutlich bessere Bewältigung der Tumorlast, erkennbar durch eine Reduktion der WT1-Transkripte im Verlauf der Therapie (p=0,057), als Patienten mit höheren PD-1-Werten zu Beginn der Vakzinierung.

Weiterhin zeigen die phänotypischen Untersuchungen auch bei der T-Zell-Population Einschränkungen bei der Entwicklung eines effektiven immunologischen Gedächtnisses. Nach dem ersten Kontakt einer naiven T-Zelle mit ihrem spezifischen Antigen reift die Zelle und exprimiert Oberflächenmoleküle, die ihr das Einwandern in sekundäre Lymphorgane erleichtern, um dort als Gedächtnisszelle zu verbleiben [8,

[9]. Eines dieser Oberflächenmoleküle, CCR7 (*cc-chemokine receptor 7*), spielt in unserem Impfschema eine wichtige Rolle. Das Patientenkollektiv zeigt im Verlauf zwei wesentliche Verhaltensweisen, welche die CCR7-Expression betreffen: bei ungefähr der Hälfte der Patienten nimmt die Frequenz an $CCR7^+$-T-Zellen deutlich ab, während sich bei den restlichen Patienten die entsprechenden Frequenzen erhöhen. Die Abnahme von $CCR7^+$-T-Zellen kann, ebenso wie die Präsenz von PD-1, mit einem negativen klinischen Verlauf korreliert werden (p=*) (Absatz 4.2).

Einfluss von immunregulatorischen Zellen auf die Therapieeffizienz

Ein weiterer wichtiger Punkt bei der Analyse von Immunresistenzmechanismen betrifft die Präsenz immunsupprimierender Zellen. Auch diese dienen eigentlich zur Hemmung einer Immunantwort nach der Eradikation eines Pathogens, können aber in der Tumortherapie einen negativen Einfluss ausüben [10-12]. Die ermittelten Daten zeigten zu beiden Zeitpunkten erhöhte Frequenzen von regulatorischen T-Zellen als auch von Suppressorzellen myeloiden Ursprungs, sogenannten *myeloid-derived suppressor cells* (MDSCs), ein Effekt, der für die AML-Erkrankung allerdings bekannt ist. Jedoch lieferte der Vergleich zwischen den beiden Zeitpunkten für keine der beiden Zellpopulationen einen signifikanten therapeutischen Zusammenhang mit reduzierter T-Zellfunktionalität oder dem klinischen Verlauf (Absatz 4.3.2.1).

Immunevasion durch Leukämische Blasten und die resultierende Interferenz mit der Therapie

Im Kontext von Immunresistenzmechanismen sollte auch immer ein Fokus auf der Immunevasion des Pathogens liegen: wenn man Immunität unter einem evolutionären Gesichtspunkt betrachtet, übt das immunologische Verteidigungssystem des Wirtes einen großen Selektionsdruck auf entartete Zellen oder andere Pathogene aus. In diesem Zusammenhang treten oft einzelne Tumorzellen auf, die durch aktive oder passive Mechanismen in der Lage sind, der spezifischen Immunabwehr zu entgehen und im späteren Verlauf ein Rezidiv oder Metastasen zu verursachen. Interessanterweise ahmen diese aktiven Mechanismen oft konventionelle immunologische Prozesse nach, die üblicherweise eine Immunantwort dämpfen. Leukämien treten hier verstärkt in den Vordergrund, da sie, ebenso wie alle Immunzellen, hämatopoietischen Ursprungs sind und deshalb

potentiell über ein ähnliches Transkriptom wie diese verfügen [13, 14]. Aus diesem Grund treten bei Leukämien häufig Membran-gebundene oder lösliche Immunsuppressiva wie TGF-β, PD-L1, IL-10, IDO, HMOX, CXCR4, VEGF, PI-9 und IL-8 auf, welche die Sensitivität der Blasten gegenüber T-Zell-vermittelter Apoptose reduzieren und/oder als negative Regulatoren auf zytotoxische T-Zellen wirken.

Vergleichende Untersuchungen an Knochenmark- oder Blutproben von Patienten, bei denen im Verlauf der Therapie ein Rezidiv auftrat, zeigten, dass die Expression der oben genannten Immunmodulatoren zum Initiierungs- und/oder Rezidivzeitpunkt innerhalb der Patienten heterogen verteilt waren und somit keinen generellen Marker auf ein im Therapieverlauf auftretendes Rezidiv beinhalten. Nichtsdestotrotz können die individuelle Präsenz und Induktion dieser Marker einen Einfluss auf den klinischen Verlauf bei einzelnen Patienten ausüben.
In Übereinstimmung mit den Ergebnissen über die PD-1-Präsenz auf T-Zellen zeigte auch die veränderte Expression des PD-1-Liganden (PD-L1) auf Blasten eine Korrelation zur mittleren Überlebensdauer (p= *; Figure 32 A): ein Teil der Patienten mit zu Beginn der Therapie stark erhöhtem Level an PD-L1 mRNA, zeigte im Verlauf der Therapie eine starke Abnahme dieser Werte. Interessanterweise traf dies nur auf Patienten zu, die am meisten von der Therapie profitierten und am längsten rezidivfrei blieben. Erklärungen hierzu sind rein spekulativ, allerdings zeigen aktuelle Studien, dass PD-L1-Level vom Reifungsstatus der Zelle als auch durch Umgebungsfaktoren wie IFN-y beeinflusst werden können [15, 16]. Zukünftige Untersuchungen sollten Fragestellungen hierzu beinhalten.
Ein weiterer interessanter Aspekt dieser Untersuchungen wird durch die gegenläufige Regulation von TGF-β repräsentiert. Während TGF-β-mRNA-Level in Patienten mit frühem Rezidiv sinken, erhöhen sich die TGF-β-mRNA-Level in Patienten mit spätem Rezidiv (Figure 32 B). Dies scheint paradox, da im Kontext eines Rezidivs TGF-β eher eine Rolle als Tumorpromotor zugedacht wird, allerdings zeigen Studien, dass eine Störung in der frühen Phase des TGF-β-Signalweges zu einer erhöhten Proliferations- und somit auch Mutationsrate von Tumorzellen führen kann [17]. Prozesse, die erst zu einem späteren Zeitpunkt mit der TGF-β Signalkaskade interferieren, führen im Gegensatz dazu, dass sich die Expansion

eines Tumors langsamer, dafür aber wesentlich invasiver vollzieht. Auch hierauf sollte in weiterführenden Studien ein Augenmerk gerichtet werden.

Resumée und Ausblick

Die ermittelten Daten zeigen, dass in unserem spezifischen Therapieansatz, der wiederholten Vakzinierung von AML-Patienten mit einem HLA-A201-restringierten $WT1_{126–134}$ -Epitop in Kombination mit GM-CSF und KLH, eine eingeschränkte T-Zell-Funktionalität einen wesentlichen Grund für die beobachtete verminderte Therapieeffizienz darstellt. Immunresistenzmechanismen leukämischer Blasten spielen hierbei keine übergeordnete Rolle, individuelle Effekte können aber nicht ausgeschlossen werden. Ebenso scheint es, dass auch die Präsenz von immunregulatorischen Zellen wie T_{regs} oder MDSCs nicht durch die Vakzinierung manipuliert wird und dass diese keinen generellen Einfluss auf die Therapieeffizienz ausüben.

Diese Arbeit, wie auch die Arbeiten anderer zeigen, dass die Krebsimmuntherapie und besonders die Tumorvakzinierung ein wichtiges, attraktives und wachsendes Forschungsfeld mit hohem Potential repräsentiert, welches allerdings, aufgrund der großen Bandbreite an Therapieformen und Tumorentitäten, durch eine hohe Komplexität gekennzeichnet ist. Der Abschluss einer wichtigen Priorisierungsstudie für Tumorantigene als auch der aktuelle Dialog über die Harmonisierung von Vakzinestudien sollten in Zukunft zu einer gesteigerten Transparenz als auch zu einem optimierten Transfer von Erkenntnissen führen, um die Tumortherapie effektiver und für den Patienten angenehmer zu gestalten.

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Abbreviations

AA	amino acid
Ab	antibody
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APC	allophycocyanin
APCs	antigen presenting cells
BFA	Brefeldin A
B-NHL	B cell Non-Hodgkin Lymphoma
BSA	bovine serum albumine
CD	cluster of differentiation
cDNA	complementary DNA
CR	complete remission
CTL	cytotoxic T cell
DC	dendritic cell
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide-triphosphate
EDTA	ethylendiamid-tetra-acedic acid
ELISA	enzyme-linked immunosorbent assay
EORTC	*European Organization for Research and Treatment of Cancer*
et. al.	*et alii* (latin: „and associates")
FACS	fluorescence activated cell sorting
FDA	U.S. Food and Drug Administration
FITC	fluorescein-isothyocyanate
FSC	forward-scatter
GAM	goat-anti-mouse
GM-CSF	granulocyte-macrophage colony-stimulating factor
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
Ig	immunoglobulin
IL	interleukin
IMCs	immature myeloid cells
IU	international units
KLH	keyhole limpet hemocyanin
LB	lysogeny broth
MACS	magnetic-activated cell sorting
MDSC	myeloid-derived suppressor cells
MHC	major-histocompatibility-complex
mRNA	messenger RNA
$NADH^+$	nicotinamide adenine dinucleotide
NCI	*National Cancer Institute*
NIH	*National Institutes of Health*
NK cell	natural killer cell
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	progressive disease
PE	R-phycoerythrin
PerCP	peridinin chlorophyll protein complex
PFS	progression-free survival
PR	partial remission
RCC	renal cell carcinoma
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
qRT-PCR	quantitative reverse-transcriptase polymerase chain reaction
SAV	streptavidin
SD	stable disease
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	side scatter
SEB	*staphylococcus aureus* enterotoxinB
SLOs	secondary lymphoid organs
TAA	tumor-associated antigen
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EFF}	effector T cell
T_{EM}	effector memory T cell
TGF	transforming growth factor
T_H1	$CD4^+$ T-helper cell, producing type-1 cytokines
T_H2	$CD4^+$ T-helper cell, producing type-2 cytokines
T_H17	$CD4^+$ T-helper cell, producing IL-17
TIL	tumor infiltrating lymphocytes
TNF	tumor necrosis factor
T_{reg}	regulatory T cell
WHO	*World Health Organization*
WT1	Wilms' tumor gene 1

1 Introduction

With the discovery of antibiotics and improving medical knowledge in diagnosis and treatment of once fatal diseases, demographic aging results in cancer being the second most common cause of death after heart disease in high-income countries in 2004 [18]. Despite encouraging advances in early diagnosis and improvements in therapy and combination therapies, cancer remains a major public health dilemma, as substantial groups of cancer patients lack effective treatment options, and even larger groups lack curative therapies [19].

Over the past two decades, advances in basic immunology as well as improved understanding of the interactions between the immune system and tumors, generated a renewed interest on Ehrlich's postulate to approach cancer treatment immunologically [20-23]. The concept of cancer immunotherapy provides an alternative perspective, since it is not associated with many of the drawbacks of conventional therapies such as chemotherapy, radiotherapy and surgery, which are rather unspecific, accompanied by more or less severe adverse effects and do, in a lot of cases, not lead to a permanent cure. Those treatments are highly active in eliminating the major tumor mass, but are less effective in eliminating residual cancer cells and in preventing relapse of the disease. These occasionally detrimental insufficiencies shift current cancer research to a more biological approach on tumor therapy:

In the early 20th century, Paul Ehrlich was the first to suggest, that the immune system plays a pivotal role in the protection against tumors [24]. Since then, the biological therapy of cancer has evolved in an impressive manner: From 1957 on, Burnet and Thomas theorized that any organism sufficiently complex and with a life span long enough to be threatened by cancer must evolutionary acquire mechanisms capable of protecting against tumors. These considerations led to the immunosurveillance hypothesis [25, 26], describing the guarding nature of lymphocytes by constantly eliminating transformed cells in immunologically intact individuals - a milestone in cancer immunotherapy. However, as many cancers are known to appear in the setting of immune/inflammatory responses [27], the concept of immunostimulation, dating back to Rudolf Virchow's work in the 19th century [28], competed with immunosurveillance on describing the cause of tumor promotion.

The proof and acceptance of Virchow's thesis on immunostimulation and it's co-existance with immunosurveillance finally led to the understanding of immune editing [20, 29], a process describing the adaption and evolution of tumor cells in regards to the selectional pressure mediated by the immune system, thereby sculpting the immunogenic phenotype of tumors as they develop. This effect reveals the complex nature of tumor immunotherapy. Briefly, cancer immunoediting comprises three processes, usually refered to as "the three Es":

a) **e**liminiation/immunosurveillance,
b) **e**quilibrium, a phase in which the immune system iteratively promotes the generation of immune-resistant tumor variants, and
c) **e**scape, a phase in which the immune-sculpted variants expand in an uncontrolled manner.

Based on this model, tumors can be specifically targeted and eliminated by the immune system (elimination), but if the immune system is not able to completely eradicate the nascent tumor, the tumor might persist but is controlled by the immune system (equilibrium). At some point at this stage, single tumor cells may transform and elude immune control (escape), thereby causing relapses [20].
However, Blankenstein *et al.* advance the view, that spontaneous tumors are indeed immunogenic, but that the resulting immune responses are unable to efficiently limit tumor growth [30]. Which of both features actually reflects the mechanisms involved in the immunesculpting of the tumor remains under strong discussion and investigation.
With the prosperous 90s, numerous achievements could be implemented in the clinical therapy of cancer: The FDA (U.S. Food and Drug Administration) approval of IL-2, GM-CSF (granulocyte-macrophage colony-stimulating factor) and the anti-CD20 antibody *Rituximab* altered the field of immunotherapy significantly [31-33]. The climax of this development was reached by Pierre van der Bruggen and colleagues who reported the first identification of a tumor-specific antigen recognized by cytolytic T cells in humans, reinforcing the idea that tumor antigens can elicit a detectable tumor-specific immune response [23]. Furthermore, with the observation, that cytotoxic lymphocytes infiltrate tumor tissue [34], are able to lyse tumor cells *in vitro* [35] and are associated with better prognosis [36] tumor therapists started to understand and actively modulate the capacity of T cell directed therapy.

Nevertheless, with the broad diversity of tumors, and them being able to potentially escape many, if not all, immunological offensives in just as diverse ways, there are still a lot of questions to be answered and obstacles to overcome while optimizing cancer treatment. In this context, the present work is thought to enlighten the understanding of immune resistance mechanisms in the setting of peptide-based vaccination therapy in acute myeloid leukemia.

1.1 Acute myeloid leukemia

Acute myeloid leukemia[a] (AML) is a relatively rare disease, accounting for a german incidence rate in adults of approximately 4.3 cases per 100 000 in men and 2.9 cases per 100 000 in women, respectively [37, 38]. It is the most common acute leukemia affecting adults, and its incidence increases with age - an important fact regarding aging populations. AML results in a massive outgrowth of leukemic blasts (Figure 1) in the bone marrow leading to a suppression of normal hematopoiesis. This usually results in a drop of red blood cells, platelets, and normal white blood cells in the peripheral blood, which could either be associated with leukocytosis, as result of leukemic blasts migrating into the peripheral blood or leucopenia, when leukemic blasts remain in the bone marrow.

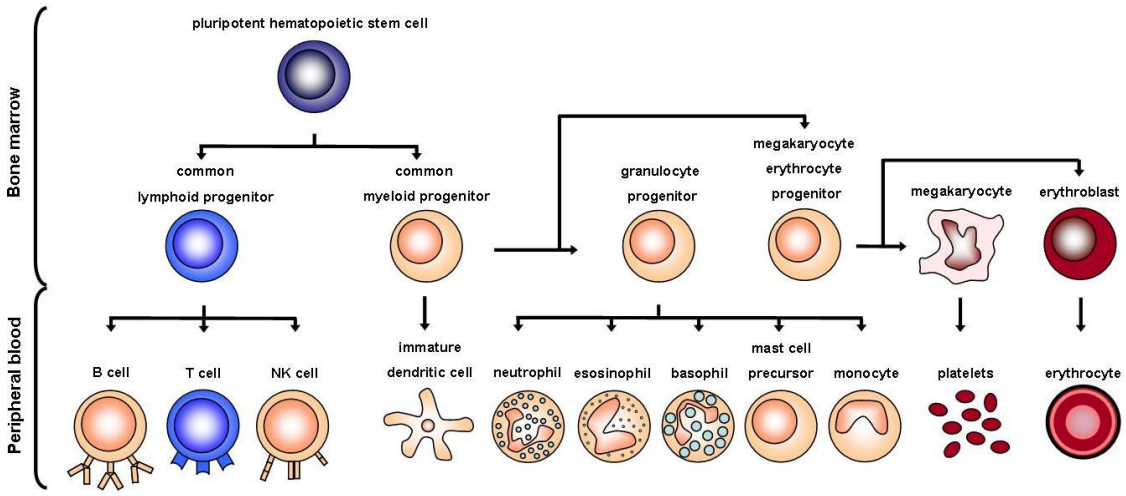


Figure 1: phylogenetic tree of the hematopoietic lineage. AML can affect any cell type derived from a common myeloid progenitor. Adapted and modified from *Janeway's Immunobiology* [39]

[a] Greek: λευκός (*leukós)* „white" und αἷμα (*haima)* „blood"

Although no specific cause of the disease could be identified yet, several risk factors for AML are known. The National Cancer Institute (NCI) lists those on its homepage[b]:

- Being male.
- Smoking, especially after age 60.
- Having had treatment with chemotherapy or radiation therapy in the past.
- Having had treatment for childhood acute lymphoblastic leukemia (ALL) in the past.
- Being exposed to atomic bomb radiation or the chemical benzene.
- Having a history of a blood disorder such as myelodysplastic syndrome.

The disease pattern may include symptoms as fatigue, shortness of breath, easy bruising and bleeding, and increased risk of infection. AML usually progresses rapidly and is typically fatal within weeks or months if left untreated due to massive infections or bleedings. AML can be classified according to the WHO [40, 41] - or the French-American-British (FAB) [42] - classification system (comparative summaries in Table 1 and Table 2).

French-American-British (FAB) Classification	
M0	Minimally differentiated leukemia
M1	Myeloblastic leukemia without maturation
M2	Myeloblastic leukemia with maturation (t(8;21)(q22;q22), t(6;9))
M3	Hypergranular promyelocytic leukemia (t(15;17))
M4	Myelomonocytic leukemia (inv(16)(p13q22), del(16q))
M4 Eo	Increase in abnormal marrow eosinophils (inv(16), t(16;16))
M5	Monocytic leukemia (del (11q), t(9;11), t(11;19))
M6	Erythroleukemia
M7	Megakaryoblastic leukemia (t(1;22))

Table 1: Acute Myeloid Leukemia Classification Systems according to the FAB. T= translocation, inv = inversion, del = deletion, p/q = structural nomenclature of the chromosome: p= short arm, q= long arm.

While the FAB divides AML into 8 subtypes M0 through to M7, based on morphological as well as on immunological criteria, the WHO-classification attempts to define biological entities, reflecting the fact that an increasing number of acute leukemias can be categorized based upon their underlying cytogenetic or molecular genetic abnormalities that form clinico-pathologic-genetic entities [41, 43].

[b] www.cancer.gov

World Health Organization Classification	
AML with recurrent genetic abnormalities	AML with t(8;21)(q22;q22);*RUNX1/RUNX1T**
	AML with abnormal bone marrow eosinophils (inv(16)(p13q22) or t(16;16)(p13;q22);*CBF*β/*MYH11*)*
	Acute promyelocytic leukemia (AML with t(15;17)(q22;q12) (*PML*/*RAR*α) and variants)*
	AML with t(9;11)(p22;q23); *MLLT3-MLL*
	AML with t(6;9)(p23;q34); *DEK-NUP214*
	AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
	AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
	Provisional entity: AML with mutated NPM1
	Provisional entity: AML with mutated CEBPA
AML with multilineage dysplasia	Following a myelodysplastic syndrome or myelodysplastic syndrome/ myeloproliferative disorder
	Without antecedent myelodysplastic syndrome
AML and myelodys-plastic syndromes, therapy-related	Alkylating agent-related
	Topoisomerase type II inhibitor-related
	Other types
AML not otherwise categorized	AML minimally differentiated
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic and monocytic leukemia
	Acute erythroid leukemia
	Acute megakaryoblastic leukemia
	Acute basophilic leukemia
	Acute panmyelosis with myelofibrosis
Myeloid sarcoma	Syn.: extramedullary myeloid tumor; granulocytic arcoma;chloroma
Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis (syn.: transient myeloproliferative disorder)
	Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm	
Acute leukemias of ambiguous lineage	Acute undifferentiated leukemia
	Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); *BCR-ABL1*_
	Mixed phenotype acute leukemia with t(v;11q23); *MLL* rearranged
	Mixed phenotype acute leukemia, B/myeloid, NOS
	Mixed phenotype acute leukemia, T/myeloid, NOS
	Provisional entity: Natural killer (NK)–cell lymphoblastic leukemia/lymphoma

Table 2: Acute Myeloid Leukemia Classification Systems according to the WHO. * Diagnosis is AML regardless of blast count; T= translocation, inv = inversion, del = deletion, p/q = structural nomenclature of the chromosome: p= short arm, q= long arm.

On this account, AMLs with cytogenic abberations are separated from therapy-induced or morphological AMLs. Consequently, the different subtypes exhibit varying prognoses and responses to therapy. Conventional treatment options for AML are limited and consist primarily of chemotherapy and/or stem cell transplantation. With standard chemotherapy, the majority of AML patients reach a complete remission, but only 20% to 40% achieve a disease-free survival of more than 5 years [44]. Chemotherapeutic treatment is generally composed of two phases: induction and postremission/consolidation therapy. The goal of the induction phase is to reach a complete remission, meaning that no disease can be detected with available diagnostic methods. This already implies that even after complete remission, small numbers of leukemic cells can still remain in the body and if no further postremission or consolidation therapy is given, almost all patients will eventually relapse. The goal of consolidation therapy therefore is to eliminate any residual undetectable disease and to achieve a permanent cure. Because of its severity, hematopoetic stem cell transplantation (HSCT) usually is only performed in high risk patients and if previous chemotherapy failed, but inclusion criteria are well defined and not all patients are candidates for stem cell transplantation.

For those patients the only curative alternative, besides palliative care, is the inclusion in a clinical trial. Current investigations include cytotoxic drugs, targeted therapies, adoptive T cell transfer and antigen-directed immunotherapy. The better characterization of graft-versus-leukemia effects after allogeneic stem cell transplantation, donor lymphocyte infusions, and identification of leukemia-associated antigens have set the headstone of immunotherapy strategies.

1.2 Tumor antigens

The term *tumor antigen* describes antigens, which are exclusively or predominantly expressed on tumors and therefore are principally adequate to induce a tumor-specific immune response. This feature appoints tumor antigens their role as major targets in cancer immunotherapy, as well as important diagnostic markers [45]. Like all other antigens, tumor antigens are presented via MHC-class I- or MHC-class II and therefore represent target structures for cytotoxic T-lymphocytes (CTLs), T-helper cells as well as B-cells [46-48].

As noted before, tumor antigens are not necessarily tumor specific: they usually are over-expressed as a result of the malignant transformation of a tumor cell, leading to

an unnatural high "concentration" of an habitually factor which has made the use of the term *tumor-associated antigens* (TAAs) more appropriate [49]. In general, tumor-associated antigens can be divided into five groups: (1) Tumor-specific shared antigens (formerly: Oncospermatogonal (Cancer/Testis)-antigens) that are expressed in a range of different tumor types but not in normal tissues except testis, (2) differentiation antigens, (3) antigens encoded by mutated normal genes, (4) self-antigens, over-expressed in malignant tissues and (5) antigens derived from oncogenic viruses [50, 51]. A short register of the most prominent TAAs is shown in Table 3.

Group	Antigen
(1) Tumor-specific shared antigens	MAGE[52], RAGE[53], NY-ESO-1[54]
(2) Differentiation antigens	MART-1/Melan-A[55], Tyrosinase[56], gp100[57]. PSA[58], AFP[59]
(3) Antigenetic epitopes of mutated genes	CDK4[60], Caspase-8[61], p53[62]
(4) Self-antigens derived from malignant tissues	HER-2/neu[63, 64], WT1[65]
(5) Viral antigens	HPV[66], EBV[67]

Table 3: Summary of the major tumor antigen-groups and some exemplary antigens.
Subgrouping according to Van den Eynde and van der Bruggen [51].

The *serological identification of antigens by recombinant expression cloning* (SEREX) [68] as well as the process of digital prediction by online databases (e.g. SYFPEITHI[c] or BIMAS[d]) [69, 70] has led to the identification and characterization of numerous TAAs thereby also mounting the number of identified tumor antigens for hematopoietic tumors. Interestingly, in the case of myeloid tumors like chronic myeloid leukemia (CML), AML or myelodysplastic syndrome (MDS), it seems that most of the TAAs associated with these diseases can be related to important functions affecting the cell-cycle and/or cell-proliferation. [71]. The high functional relevance of these TAA reduces the risk of induction of TAA-loss as tumor-escape variants.

1.3 Targeted immunotherapy and WT1

In contrast to all other standard modalities (surgery, chemotherapy, radiotherapy, and adaptive immunotherapy), an effective vaccine-induced immune response against

[c] http://www.syfpeithi.de/
[d] http://www-bimas.cit.nih.gov/molbio/hla_bind/

tumor uniquely may bear the potential to last a lifetime [72]. The rationale of therapeutic cancer vaccines is to "teach" the patient's immune system to specifically recognize and eliminate tumor cells. When fully activated the immune system has immense potential, as is apparent from several autoimmune diseases (*e.g. lupus erythematosus*[73]*, psoriasis*[74] *and pemphigus vulgaris*[75]), mismatched transplanted organs undergoing rapid immunological attack and rejection, as well as, in opposition to the later, graft-versus-host disease (GvHD) [76]. These examples demonstrate, that one of the most important features of an intact immune system is to differentiate between "self" and "non self". In the case of external pathogens like bacteria, viruses or transplants this system usually works efficiently, but due to the fact that tumors normally represent derivatives from mutated "self"-cells, the immune-system may be constricted in its activity.

The tight discrimination between tumors and normal tissue by the immune system is therefore crucial for the eradication of the tumor and the survival of the affected individuals. To support the immune system in this task, the recognition of tumor cells can be primed and facilitated by adaptive and/or active immunotherapy. In contrast to adaptive immunotherapy, in which *ex vivo*-generated target specific cells or antibodies are reinfused or administered, active immunotherapy is characterized by the application of vaccines that aim at eliciting a specific immune response against over-represented tumor antigens *in vivo*. [72].

1.3.1 Tumor vaccination

Vaccination[e] represents a very potent and successful strategy to treat or prevent diseases. Intentionally used to describe the injection of smallpox vaccine, the term today refers to a general administration of antigenic agents to stimulate adaptive immunity to recognize a specific antigen and to establish an immunological memory [4].

Indifferent of the vaccine-approach, the common feature of all represents the delivery of an antigenic structure that can be uptaken and be presented by antigen-presenting cells (APCs) via MHC molecules. The recognition of these peptide/MHC-complexes by T cells potentially leads to the stimulation of the antigen-specific lymphocytes as well induces further co-stimulatory signals for a proper activation of

[e] from the latin word *vacca* – cow. The term was first used by Edward Jenner, a reference to the immunization for smallpox by inoculation of humans with cow-pox virus.

the adaptive immune system. Ideally, this cascade results in the generation of immediate effector T cells, as well as in a permanent reservoir of memory T cells which provide long-term protection upon second encounter with the same pathogenic structure. In cancer immunotherapy, this is accomplished by the usage of defined TAAs or tumor lysate applied as mainly cell-based vaccines, DNA- or RNA-based vaccines, vector-based vaccines and protein- or peptide-based vaccines. To achieve a better stimulation the antigen is usually combined with adjuvant immune stimulators or reponse modulators. Independent of the technique, all strategies aim at the induction of an endogenous, long-lasting tumor response similar to those in antiviral or antimicrobial vaccine-settings.

Cancer vaccination can either be therapeutic or prophylactic, treating existing cancer or preventing either infections with cancer-causing viruses or the development of cancer in certain high risk individuals, respectively.

Various strategies for therapeutic cancer vaccines have been proposed to elicit immune responses against tumor antigens, including cell-based vaccines, DNA- or RNA-based vaccines, vector-based vaccines, and protein- or peptide-based vaccines (Figure 2) [77, 78].

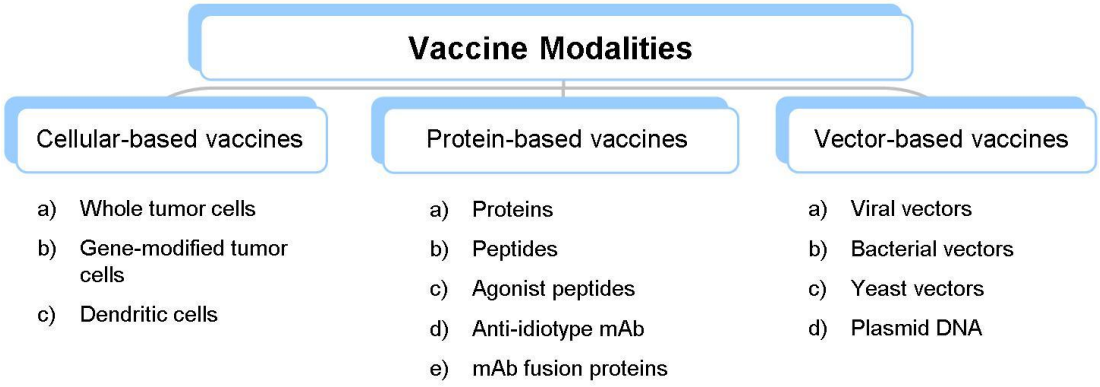


Figure 2: General vaccine modalities. Three main vaccination types are totally available against cancer such as cellular-based vaccines, protein-based vaccines and vector-based vaccines. Each these types divide into the subgroups in detail. Among them, DNA vaccines and protein/peptide vaccines have been further involved in vaccine design. Adapted and modified from Bolhassani *et al.* [78]

In the past years the efforts to establish preventive vaccines targeting tumor-inducing viruses have been successful and vaccines against human papilloma virus (HPV) [79] and hepatitis B virus (HBV) [80] associated cancers, are well established.

In contrast to well-established microbial or virally induced diseases, a real proof of efficacy for anti-tumor vaccines has only be demonstrated for a very few disease settings. In contrast to external pathogens, the major hurdle in developing efficient

cancer vaccines is represented by the lack of appropriate and distinct tumor-specific antigens and the weak immunogenicity displayed by tumor-associated antigens, as inherited by all self-antigens. Additionally, the immunotherapeutic targeting of a shared antigen aims at breaking otherwise exhibited tolerance and to induce autoimmunity, measurements that increase the curative complexity.

Besides the natural obstacles, the huge variety of antigens, the different systems used to deliver the vaccine, as well as the use of different adjuvants to costimulate specific compartments of the immune system, limit the ability to discriminate and to compare the different clinical trials.

In the context of already manifested tumors, the failing induction of effective and long-lasting tumor-specific immune responses remains the most intriguing point. Up to date, only one vaccine with proven benefit in hormone-refractory prostate cancer is FDA-approved and commercially available [81].

Therefore, the current formation of initiatives to facilitate and to structure cancer-vaccine research possibly provides a catalytic boost to the implementation of immune therapeutic cancer-vaccines in the clinic in a near future [82].

1.3.2 Protein- and Peptide-based vaccination

As briefly mentioned in section 1.3.1, vaccination with peptides, besides cell-based or nucleic acid-based vaccines, represents a very interesting and prospering approach of active tumor therapy. Peptides play critical roles in determining the magnitude and specificity of cell-mediated and humoral immunity, and can contain the minimal sequences necessary for immunomodulation. Furthermore, peptides can be easily synthesized and characterized, and are generally more stable than whole pathogens or full-length proteins [83].

The settings cover a broad spectrum of regimens, including single agents or combinations of proteins, heat-shock proteins (HSPs), peptides and agonistic peptides, anti-idiotype vaccines and fusion proteins [72, 84-87]. In contrast to cell-based vaccines, production and storage of peptides are feasible and administration needs no time-consuming preliminary preparations. Additionally, as tumor cell lysates contain different self-proteins with no therapeutic benefit that are all potentially capable of generating an autoimmune response, the particular administration of specific TAAs prevents unwanted side-effects [72]. The clinical response towards peptide based trials

is generally determined by the induction of TAA-specific cytotoxic T-lymphocytes and/or the production of cytokines in the $CD4^+$- and $CD8^+$-T cell compartments.

Under ideal conditions, vaccination leads to the activation of both, effector and memory T cells. The administered peptides usually are MHC-class-I restricted, stimulate $CD8^+$ effector T cells and fail to deliver additional activation signals to T-helper cells for appropriate CTL-costimulation. In the last few years a few MHC-class-II-binding epitopes could be identified, but as they lack for the specific indication, the co-administration of immunogenic adjuvants, that induce an antigen-unspecific T-helper response, is very common. The consecutive lysis of tumor cells is thought to release additional antigenic material, delivering additional triggers to a tumor-related immune response which can be assessed by the humoral response of the patients' immune system [88-91].

In the same context, the use of immunogenic adjuvants produces an interesting side effect, as several groups have described an induction of specific immunoglobulins not only for the TAA-peptide, but also for the adjuvants used in different therapeutic settings [92]. This immunogenic effect permits a direct comparison between the developments of specific immune responses towards different kinds of antigens in the same setting.

However, the use of specific proteins or peptides as targets for immunotherapy clearly requires a careful choice of the targeted TAAs and their epitopes, as peptide based vaccination also needs to overcome some methodical obstacles: As the nature of TAAs classifies them as self-proteins, they may exhibit a generally weak immunogenicity especially when using a single epitope [72]. Further, tumors can easily escape immune recognition through antigen mutation or loss. In addition, the use of peptides is usually HLA-restricted (*e.g.* epitope-based vaccines) and therefore limited to a specific subset of patients [2, 93]. Therefore, the ideal TAA should be widely expressed in different tumor types and also play a pivotal role in the oncogenic processes or in cancer cell survival, to avoid immune escape by mutations or loss of antigens by tumor cells [72, 82].

1.3.3 Vaccine adjuvants

As noted before tumor vaccination has been clinically tested and has been constantly improved over the past two decades. In general, current cancer vaccines are composed out of three parts: the antigene(s), adjuvant(s) and a delivery/application

system. This composition is consistent with the actual development of common vaccines, shifting from live attenuated or inactivated pathogens towards well-defined and purified antigenic substructures, which possess a bettter safety profile and can be produced in bulk quantities [94]. Nevertheless, this recent development requires the co-administration of immunmodulative adjuvants as these artificial antigenic substructures lack additional pathogenic compounds that enhance immunity by efficiently activating several components of the innate immune system when using live attenuated or inactivated pathogens [95, 96].

The administration of adjuvants is a heterogenous field, including vaccine designs using single agents or combinations of different adjuvants depending on the effect the have to elicit. In the context of MHC-class-I restricted peptide settings, adjuvants are necessary to stimulate, activate and to recruit T-helper cells (T_H). On this account, traditional methods use larger carrier proteins, such as keyhole limpet hemocyanin (KLH)[97]; a metalloprotein isolated from the mollusk *megathura crenulata*, or diphteria toxoid [98] that both provide sources of T_H-cell epitopes. In current MHC-class-I restricted peptide vaccinations in AML, actual adjuvants besides TAA-specific MHC-class II epitopes, comprise CpG 7990 [99], a TLR9 agonist oligodeoxynucleotide, Montanide [2, 65], a water in oil emulsion, or GM-CSF [93, 100, 101]. In our setting, the MHC-class-I restricted peptide was administered in a combination of KLH and GM-CSF [93] (more detailed description in section 1.3.4).

Concerning the delivery of antigenic peptides and adjuvants many designs and systems have been explored [78]. Generally, besides soluble antigens, delivery systems can be allocated to one of two substes, live vectors or non-living delivery systems. The latter ones, such as alum, liposomes, emulsions, immune stimulating complexes (ISCOMs) or biodegradable microparticels attempt to mimic the particulate nature of pathogens, by incorporating the antigen into particles which are retained in the body longer than soluble antigens and are more easily taken up by APCs. In contrast, live vectors, such as vaccinia virus, adenovirus and BCG (*Bacillus Calmette-Guérin*) represent antigenic structures in its native conformation from a relevant pathogen and thereby provide a co-stimulatoy immune-boosting effect [78, 102]. The appropriate choice therefore depends on therapy goals, immunologic circumstances as well as suitability in the therapeutic context.

1.3.4 WT1 as therapeutic target

The embryonic transcription factor WT1 has attracted considerable interest as target antigen for T cell based immune therapies[103] and was recently listed on position one in the ranking of the *cancer antigen pilot priorization* of the National Cancer Institute[82], attempting to compare current TAAs according to predefined and preweighted criteria of an "ideal" tumor antigen.

WT1 was first described in 1990 as a tumor suppressor gene associated with Wilms tumor (a nephroblastoma)[104], but also acts as an oncogene in multiple malignancies[105]. Currently, both labels are thought to be inadequate in the WT1 context, as, rather than describing the cellular function of *WT1*, they more accurately describe the effect of *WT1* alteration in the varying contexts of cell type, differentiation status, presence of other gene alterations and microenvironment. On this account, WT1 probably can be best described as a chameleon-like gene, functioning as either a tumor suppressor gene or an oncogene, depending on the cellular context [106].

The *WT1*-gene product encodes a transcription factor of the zinc-finger family (Figure 3) , which plays a key role in proliferation, apoptosis and organ development[107]. In adults WT1 expression is normally limited to the urogenital system, the central nervous system and to hematopoietic tissues, but pathological over-expression of the *WT1* gene occurs in hematological malignancies and various types of solid tumors [103, 108-110].

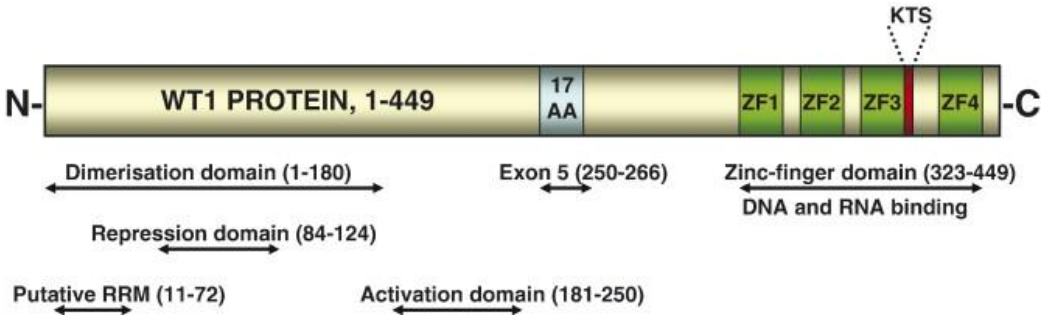


Figure 3: Schematic representation of the WT1 protein. The N-terminal domain comprises a dimerisation domain; a transcriptional activation and repression domain; and a putative RNA Recognition Motif (RRM). Alternative splicing of exon 5 and the amino acids KTS generates four main WT1 isoforms. [111]

First evidence of WT1 involvement in leukemia was reported in 1990 by Call *et al.* [104] in a pre-B cell line, as well as by others, initially appointing WT1-overexpression to 14-68 % of acute leukemia cells [112, 113]. Up to date WT1-overexpression is considered to virtually occur in all subtypes of adult ALL and

AML [114]. Depending on the source, expression frequencies in AML range from 67% up to 89% [115-117]. Elevated WT1-mRNA- levels are associated with short time transformation of MDS to AML [118] and also are a strong indicator of increased relapse frequencies after chemotherapy due to minimal residual disease [119, 120]. Interestingly, the majority of acute leukemias do not exhibit mutations of the WT1 gene, suggesting that the wild-type protein may play an important role in leukemogenesis [121]. Mutation rates in AML comprise values ranging from 5-12 %, showing higher mutation frequencies in patients with younger age [122, 123].

In vitro and *in vivo* evidence proved that humoral immune responses against the WT1 protein can be elicited in patients with WT1-expressing hematopoietic malignancies [124]. The same group recently showed, that high values of naturally occurring anti-WT1-antibodies are a strong predictor of longer survival in patients with MDS or MDS-derived AML[125] and are associated with favorable prognosis in non-small cell lung cancer (NSCLC)[126]. In contrast to differentiation antigens, which are mainly used as targets in melanomas and other solid tumors, WT1 is a crucial factor concerning the proliferation of the tumor. In fact, experiments using WT1-anti-sense oligonucleotides demonstrated reduction of proliferative capacity and differentiation of leukemic blasts and tumor cells *in vitro* [127]. Due to the functional relevance of WT1, the progression of WT1-negative tumor derivates, in the context of immune escape, seems unlikely. This is consistent with data generated in our institution, showing that loss or mutation of WT1 occurs in only one out of ten patients [128]. To date several MHC-class-I- as well as MHC-class-II-restricted peptides, binding to HLA-A1, HLA-A0201 and HLA-A2402 or HLA-DR04 and HLA-DR53, respectively, have been identified as T cell epitopes for WT1 (Table 4).

MHC-subtype	**Epitope**
HLA-A1	TSEKRPFMCAY ($WT1_{317-327}$)
HLA-A0201	RMFPNAPYL ($WT1_{126-134}$) SLGEQQYSV ($WT1_{187-195}$) CMTWNQMNL[1] ($WT1_{235-243}$)
HLA-A2402	CMTWNQMNL[1] ($WT1_{235-243}$) RWPSCQKKF ($WT1_{417-425}$)
HLA-DR0401	PQQMGSDVRDLNALL[2] ($WT1_{124-138}$)
HLA-DR53	PQQMGSDVRDLNALL[2] ($WT1_{124-138}$) EDPMGQQGSLGEQQ ($WT1_{247-261}$)

Table 4: natural WT1-derived CTL epitopes eliciting WT1-specific CTLs. [1, 2] These peptides elicit T cell responses in the context of two HLA subtypes: [1] the HLA-A201 and the HLA-A2402 subtype. [2] the HLA-DR4 and the HLA-DR53 subtype. According to Oka *et al.*[129], Asemissen *et al.*[130], Knights *et al*[131]. and Kobayashi *et al.*[132]

The first clinical vaccination trials using WT1 were initiated 2002 in our institution [93, 133, 134], as well as in the group of H. Sugiyama in Japan [2] and in the group of K. Rezvani in the USA [3, 135]. All trials showed high immunogenicity in terms of an induction of WT1-specific T cells, molecular efficacy and clinical efficacy of WT1-peptide vaccination.

The Japanese design included 12 AML patients in CR and 2 MDS patients which were vaccinated with different amounts (0.3 mg; 1 mg and 3 mg) of an HLA-A24-restricted $WT1_{(235-243)}$-epitope emulsified in Montanide, while the American study included 5 AML patients in CR and 2 MDS patients who received an HLA-A201-restricted $WT1_{(126-134)}$ -epitope in combination with an HLA-A201-restricted Proteinase-1 $(PR1)_{(169-177)}$-epitope, Montanide and GM-CSF.

In our institution, patients received 62.5 µg GM-CSF on days 1-4 and, on day 3, 0.2 mg HLA-A0201-restricted $WT1_{126-134}$ peptide admixed with 1mg KLH (Figure 4).

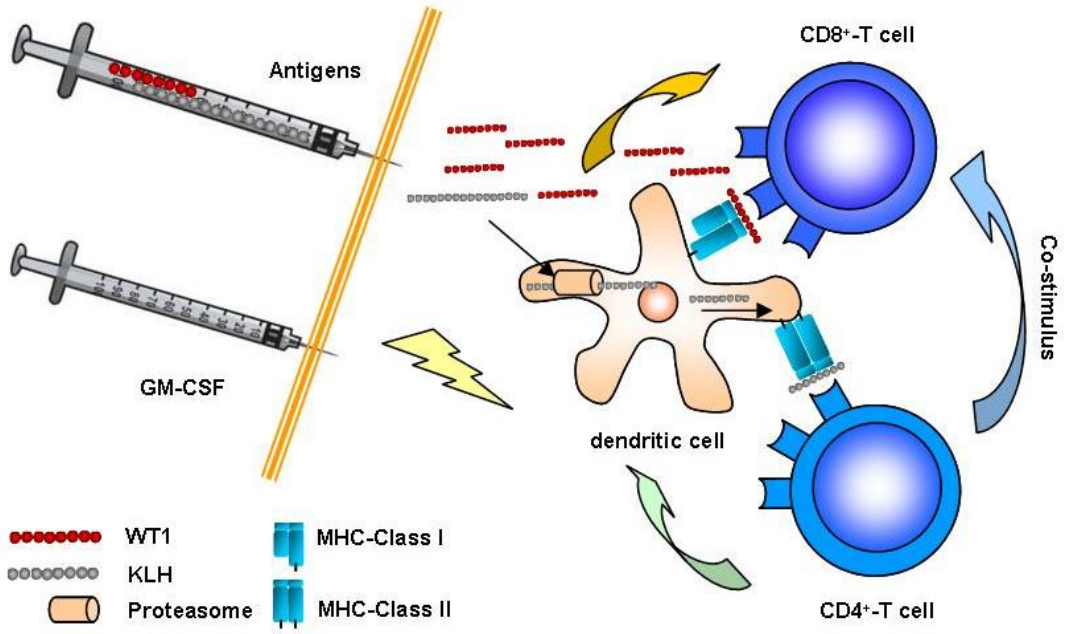


Figure 4: vaccination scheme employed in the current clinical trial at our institution. Patients received GM-CSF on days 1-4 to chemotactically attract DCs to the injection site. On day 3 the $WT1_{126-134}$ peptide, which competitively binds to MHC-class I molecules to be recognized by specific CTLs was injected. Together with the WT1 epitope, the whole protein KLH was administered, which is taken up pinocytotically by the DCs, internally processed and presented via MHC-class II molecules to deliver a $WT1_{126-134}$ -independent $CD4^+$-helper cell co-stimulus at the site of vaccination.

In our trial and as previously published, 17 AML-patients with active AML and 2 MDS patients with refractory anemia with excess blasts (RAEB) and without further conventional treatment option received a median of 11 vaccinations. During vaccination course one complete remission (CR, 514 days) and 14 stabilizations of disease status (SD, 101 to 1682+ days) were observed, including 4 SD coincided by a

reduction of blast count of more than 50% and 3 SD displaying hematological improvement. Up to date, two patients are still in ongoing SD for more than 1600 days and are vaccinated and monitored on a regular basis (Figure 5).

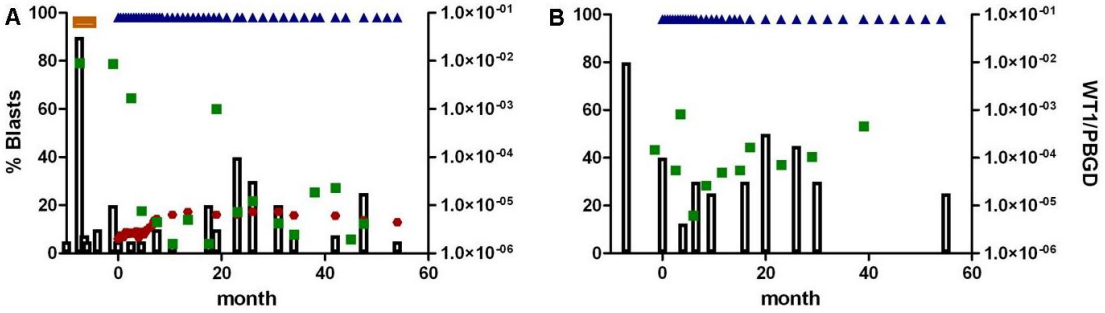


Figure 5: Long-term monitoring of patient 22 (A) and patient 23 (B) during vaccination course. Both patients are in SD since treatment initiation. □= blasts; ▲= vaccination; ■= chemotherapy; ●= hemoglobin (mM); ■ WT1-mRNA.

In addition, out of 8 AML patients in complete remission but at a high risk for early relapse, 4 patients exhibited an unexpected long progression-free survival with 10, 17, 23 and 24+ months (so far unpublished results). In 52% of the patients with active leukemia, the WT1-mRNA level in peripheral blood (PB) decreased to at least one third compared to baseline at day 0. The number of patients with induced $WT1_{128\text{-}134}$-specific T cells in PB increased from 28% at baseline to 52% in week 10 (Figure 6), whereas $WT1_{126\text{-}134}$-specific cytokine responses in patients increased from 24% to 48% in the same time frame.

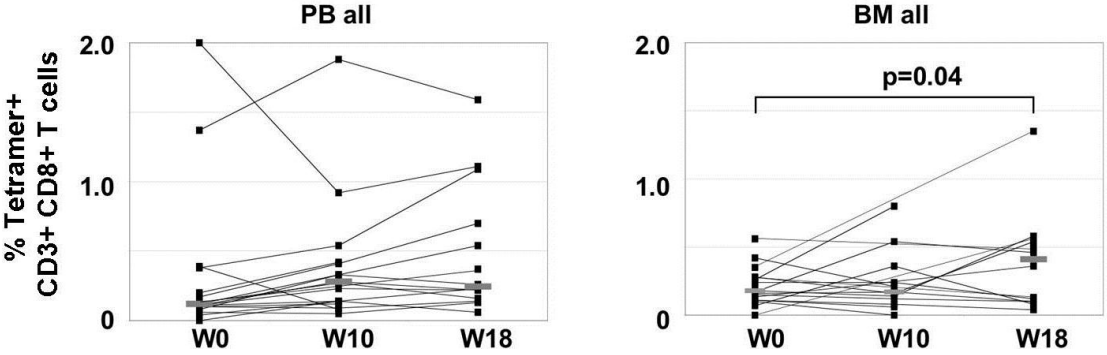


Figure 6: Representation of $WT1_{128\text{-}134}$-specific T cell frequencies in PB and BM during vaccination. Frequencies in PB increased from initial 28% at baseline to 52% in week 10 according to WT1-Tetramer staining. In BM, the actual site of the tumor, $WT1_{128\text{-}134}$-specific T cell frequencies significantly mounted in week 18 (p=0.4). Positivity was determined as % WT1-Tetramer^{+} ≥ 0.3 (mean + 2-fold standard deviation of healthy controls). Published by Keilholz *et al.* [93].

In AML patients in complete remission, the WT1-mRNA level in peripheral blood decreased in 57% of the patients. The number of patients with $WT1_{128\text{-}134}$-specific T cell increased from 28% at baseline to 71% in week 10. As 42% of these patients already exhibited $WT1_{126\text{-}134}$-specific cytokine responses at baseline, a vaccine-induced cytokine response only could be observed in one additional patient.

Considering the diverging numbers of the patients' subsets, both collectives seem to display similar vaccine-related effects concerning a molecular and cellular response in PB, suggesting a broad therapeutic benefit of $WT1_{126-134)}$-peptide vaccination.

These data correlate well with the Japanese and American trials. The Japanese group observed an induction of WT1-specific T cells in 9 out of 13 patients in PB as well as a reduction of WT1-mRNA transcripts in 5 out of 14 patients in terms of a molecular response [2]. Similar results were published by Rezvani *et al.,* observing an induction of a temporary WT1-specific T cell response in 5 out of 8 patients and a temporary molecular response in 3 out of 6 patients after a single administration of a WT1-epitope combined with a PR1-epitope, Montanide and GM-CSF in their vaccine setting [3]. Nevertheless, in a current publication Rezvani et al. showed that repeated PR1 and WT1 peptide vaccination in Montanide-adjuvant fails to induce sustained high-avidity, epitope-specific $CD8^+$ T cells in myeloid malignancies [135].

These results represent preliminary evidence of immunological, molecular and clinical efficacy of WT1-peptide vaccination in asubset of patients. Despite these encouraging results and ongoing efforts in inducing TAA-specific T cells, objective clinical responses are not reliably induced [136]. As an explanation for the lack of clinical effectiveness in the presence of antitumor T cells, a variety of mechanisms have been subject of scientific dialogue and research: the induction of tolerance towards „self"-antigens and/or the extensive immune evasion of the tumor [137-139].

In most cases, the generation of an immune suppressive microenvironment by the tumor displays a major obstacle in eradicating the tumor. This can be effected by substrate deprivation or the secretion of chemo-attractants, leading to the migration and accumulation of *e.g.* immune regulatory cells (regulatory T cells, myeloid-derived suppressor cells (MDSC)) at the tumor site[140].

Additionally and very characteristic for tumors derived from the hematopoietic system, the expression of immunomodulatory or apoptotic factors by the blasts may constitute a major obstacle for an effective immune response [141, 142].

Briefly, if specific antitumor T cells are present and the tumor still progresses, one can logically deduce three main reasons: Impairment of the effector-function of T cells, the induction of immune regulatory cells and/or immune escape mechanisms of the tumor.[f]

[f] The author is aware that the immune system represents a complex and tightly cross-linked system, in which *actio et reactio* lead to reciprocal interplay of these mechanisms.

1.4 Immune evasion and tolerance

The confrontation of the immune system to a new pathogen usually leads to one of three basic outcomes: early, complete expulsion of the foreign pathogen; overwhelming infection with failure of control; or persistence with potential long-term carriage or induction of disease. It is the last scenario in which immune evasion takes place [143].

If we gaze at immunity in an evolutionary context, the organisms' defensive resistance constitutes a formidable selection force on malignant cells or pathogenic agents. In this context, the main mechanisms of immune escape usually comprise dormancy (minimizing antigen expression), sequestration (occupying immune privileged sites), antigenic variation and counter mechanisms (see also section 1.4.1). On this account, microbial organisms as well as parasitic protozoa have coevolved with their hosts to overcome protective host barriers and, in selected cases, actually take advantage of innate host responses [144].

Nevertheless, without immune evasive mechanisms, beneficial symbiosis, like the colonization of the gastrointestinal tract or on the skin by commensal bacteria ("normal microflora") would not have taken place [145].

1.4.1 Tumor characteristics and immune escape

Despite the beneficial effects of immune evasion in symbiosis, similar effects are able to impair tumor eradication: Tumors progress in individuals because they are able to overcome immune surveillance. In the context of evolution, the tumor has to adapt to a hostile environment to escape from rejection and elimination by the immune system. Its performance depends on the ability to modulate the specific immune response or to prevent recognition by the immune system. Different tumors react different to the immunological menace, but the mechanisms used can usually be classified to one of five main strategies (summarized in Table 5) [139, 146].

However, as most of the current evidence of negative regulatory mechanisms has been acquired in the field of solid tumors, the role of the same or different mechanisms has been underexplored in hematologic malignancies. Due to the differential provenance and growth patterns of solid and hematologic malignancies, immune mechanisms explored in solid tumors may be used as guidelines, but awareness of variances in the repertoire of immune evasion in each entity may be

raised. Therefore, the understanding of the inhibitory pathways in hematological malignancies is of great importance in establishing successful cancer immunotherapy of leukemia patients in the clinic.

Strategy	Mechanism
Impaired antigen presentation	Mutation or downregulation of tumor antigens
	Mutation or downregulation of MHC genes
	Defects in antigen processing
Immunosuppressive factors	Cytokines (TGF-β, IL-8, IL-10)
	Prostaglandines
Tolerance induction	Anergy induction (PD-L1)
	Immune deviation
	Regulatory T cells (IDO)
	T cell deletion
Resistance to apoptosis	Expression of anti-apoptotic molecules (PI-9)
	Downregulation and mutation of pro-apoptotic molecules
Counteraction	Expression of death-receptor ligands (FasL, TRAIL)

Table 5: Summary of the main cancer strategies used to interfere with immune surveillance.

Interestingly, several escape mechanisms mimic or induce classical immunological processes to silence the immune response. Especially, in the context of leukemia, this effect is intensified due to the hematopoietic origin of the tumor, frequently resulting in expression of membrane-bound or soluble immunosuppressives as TGF-β, PD-L1, IL-10, IDO, HMOX, CXCR4, VEGF and IL-8 or diminished sensitivity towards apoptosis by the expression of cytolysis-inhibitors like PI-9[5, 137, 147-152].

1.4.2 Transforming-growth-factor beta

Transforming growth factor β (TGF-β) is a regulative protein, controlling proliferation and cellular differentiation in most cells. First described in the early eighties [153] as initiator of anchorage-independent growth of epithelial cells, it was found to be a regulator of the cell cycle and to be critical for wound healing in physiological conditions.

In the oncogenic context, it plays a dual role. It can serve as both, a suppressor and a promoter: TGF-β inhibits the growth of some cancers while stimulating growth in advanced cancers. Under normal conditions, TGF-β arrests the cell cycle at the G1 stage to stop proliferation, induce differentiation, or promote apoptosis through the Smad- or non-Smad-pathways (

Figure 7). The ransformation of a normal cell into a cancer cell can affect parts of the TGF-β signaling in a way that the regulative functions are shut down. These cancer cells than proliferate and induce proliferation in the tumor microenvironment by increasing their production of TGF-β. This causes immunosuppression and angiogenesis, which makes the cancer more invasive [154]. TGF-β also converts effector T cells into regulatory T cells and is also linked to T_H17 development and function [155].

However, TGF- β is also known to suppress tumor growth. Lack of, or downregulation of TGF- β receptor or SMADs is often correlated to a worse prognosis and in several murine models deletion or downregulation of TGF- β signaling results in more malignant tumor phenotypes [156, 157]. The exact underlying mechanisms driving TGF- β to either promote or suppress tumor growth are not fully understood, but are in the focus of current investigations on the modulation of the host immune system by the tumor microenvironment.

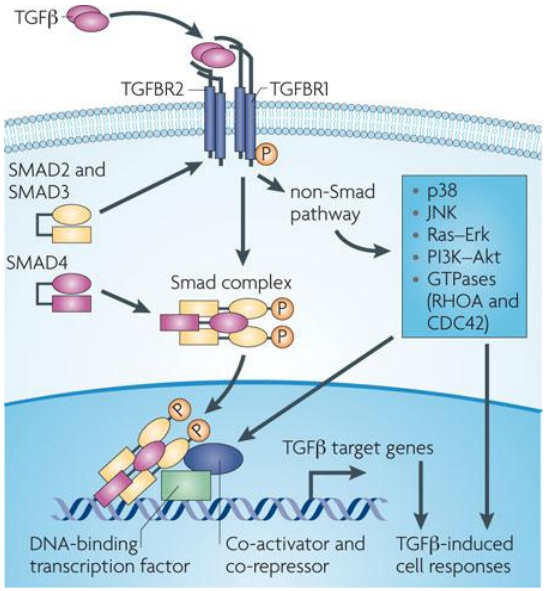


Figure 7: Transforming growth factor-β signaling. Binding of TGF-β to its receptors leads to the activation of the Smad complex which interacts with other DNA-binding transcription factors to regulate the transcription of target genes. Adapted from Ikushima *et al.* [158].

1.4.3 Programmed death receptor ligand 1

Programmed death receptor ligand 1 (PD-L1, B7-H1, CD274) is one of two ligands binding the anergy receptor Programmed Death 1 (PD-1, see also section 1.5.2). Under normal conditions, PD-1/PD-L1 interactions contribute to the maintenance of peripheral tolerance to self-antigens. Ligation of PD-L1 with PD-1 has been described to negatively regulate cytokine production and proliferation of T cells [159], as well as driving T cells into an anergic state. The major mechanism of PD-1/PD-L1 mediated T cell inhibition lies in the ability of the PD-1 receptor to recruit and activate intracellular phosphatases, which negatively regulate TCR signaling and

effector T cell responses. PD-L1 is broadly expressed in tissues and can also be found on almost all tumor entities. This fact leads to hypothesis that tumors escape from the host immune system by negatively interfering with tumor-specific T cell responses via the PD-L1/PD-1 pathway [160]. In accordance with this hypothesis, blockade of PD-L1 inhibits tumor growth or delays progression in multiple murine models [161] and PD-L1 expression on tumor cells correlates with a worse clinical outcome in several solid human malignancies [15, 162]. Despite its effects on hematopoietic cells, the role of PD-L1 in leukemia is only investigated marginally, and the few findings are controversial [163, 164].

1.4.4 Interleukin-10

Interleukin-10 (IL-10), a type-II cytokine, is produced by monocytes and to a lesser extent by lymphocytes (primarily T_H2, T_H17 and T_{regs}) [165]. It is known to transmit pleiotropic effects in immunoregulation and inflammation, by down-regulating the expression of T_H1 cytokines (*e.g.* IFN-γ, IL-2, TNF-α), MHC class II antigens and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production.

IL-10, acting through the JAK/STAT-pathways, has also been associated with tumor progression: Besides the capacity of IL-10 to induce immune-modulatory cells, the amount of tumor-derived IL-10 correlates with disease-severity and prognosis in many tumors [166]. Due to its biological function, IL-10 serves as a potent inhibitor of antigen-presentation, mainly by down-regulating MHC-class II and the interference with the CD28-pathway. This results in T cell anergy [167], but only in T cells that need co-stimulation due to low TCR-triggering. As TAAs do usually not represent a strong trigger and can additionally be down-regulated in an immune evasive context, tumor-specific T cells are potentially affected.

1.4.5 Indoleamine-2, 3-dioxygenase

Indoleamine-2,3-dioxygenase (IDO) catalyzes the limiting step of tryptophan catabolism along the kynurenine pathway (Figure 8) [168]. It has been described to play a role as a normal, endogenous mechanism of peripheral tolerance and immunosuppression in a number of immunological privileged sites such as maternal tolerance towards the fetus, the brain and the eye [169]. IDO can be expressed by

various cell types as response to inflammation, but it seems that some subsets of APC preferentially express IDO after humoral or cellular stimulation.

Still, IDO-competency of these cells is dependent of additional factors, regulated by maturation and activation signals [170]. In the context of immune tolerance, and as with any beneficial factor, tumors have adapted to express IDO to impair tumor specific immune responses [171, 172]. IDO expression on tumors occurs constitutionally, or after induction by inflammatory mediators, such as IFN-γ.

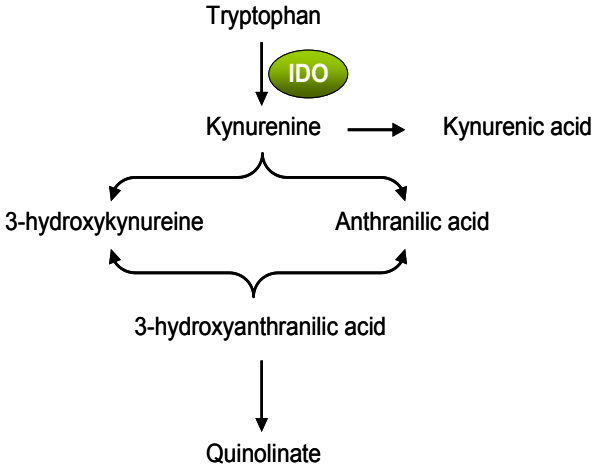


Figure 8: flow-chart of the kynurenine pathway. IDO-upregulation leads to local tryptophan-depletion at the tumor site and inhibits proper T cell function

The processing of tryptophan by IDO leads to two immunosuppressive effects, a reduction in local tryptophan and an increase in tryptophan-metabolites. The deprivation of tryptophan causes cellular stress and anergy in T cells, due to impaired tRNA-charging and in consequence impaired translation. In contrast, the increase of some tryptophan derivatives causes cell cycle arrest and apoptosis in T cells [173].

Furthermore, in an hematological context, bone marrow (BM)–derived mesenchymal stem cells (MSCs) expressing IDO have the capacity to suppress T cell responses to autoantigens and alloantigens [174]. In the case of AML, data demonstrated functional expression of the active form of IDO protein, resulting in the inhibition of T cell responses through the expansion of regulatory T cells [175].

1.4.6 Heme-oxygenase 1

Heme-oxygenase-1 (HO-1), a highly conserved molecule across almost all forms of life, catalyzes the first, rate-limiting step in the oxidation of heme, producing equimolar quantities of carbon monoxide (CO), iron ions (Fe^{2+}) and biliverdin [176]. It participates in maintaining cellular homeostasis and plays an important protective role in the tissues by reducing oxidative injury, attenuating the inflammatory response, inhibiting cell apoptosis, and regulating cell proliferation. Many data suggest that overexpression of HO-1 may defend tissues and organs from immune-

mediated injury, either through protection against oxidative damage or *via* a local immunomodulatory influence on infiltrating inflammatory cells [177].

In contrast to its isoformes HO-2 and HO-3, HO-1-expression is inducible and higher levels of HO-1 are detectable in tumors, if compared to the surrounding tissue [178]. Interestingly, it seems to be tumor dependent, whether the tumor itself or tumor-infiltrating macrophages induce the expression of HO-1. In the context of immune evasion, the biological function of HO-1 prevents the fast proliferating tumor to suffer from oxidative stress[179]. Simultaneously, elevated HO-1 levels, *i.e.* the increased concentration of CO, have anti-proliferative effects in T-lymphocytes, resulting in diminished efficacy of tumor-specific T cells [180].

Additionally to its anti-oxidative action, HO-1 can also affect cell viability by blocking apoptosis and lead to a considerable resistance of tumors to chemotherapeutic treatment [181, 182].

1.4.7 CXC chemokine receptor 4

CXC chemokine receptor 4 (CXCR4, also called fusin) is an alpha-chemokine receptor specific for stromal-derived-factor-1 (SDF-1), enhancing chemotactic activity for lymphocytes upon ligation. CXCR4 gained prominence due to its employment by HIV to infect CD4+ T cells [183].

The binding of CXCL12 to CXCR4 initiates divergent signaling pathways which can result in a variety of responses such as increased intracellular calcium-release and gene transcription leading to enhanced chemotaxis, cell survival and/or proliferation, increase [184].

In various types of cancer, including leukemia and breast cancer, CXCR4 has been identified to be a prognostic marker, mainly up-regulated in metastasis, triggering enhanced signaling and trafficking [185]. In AML, the SDF-1/CXCR4 pathway is responsible for the retention of leukemic blasts in the bone marrow [186], in which tissue specific factors, such as SDF-1 and interleukin 6 (IL-6) supplementary mediate, survival, and proliferation of tumor cells, providing a favorable and prosperous tumor-microenvironment.

1.4.8 Serpin peptidase inhibitor, clade B (ovalbumin), member 9

Serpin peptidase inhibitor, clade B (ovalbumin), member 9 (SERPIN B9 or PI-9) is a potent intracellular inhibitor of human granzyme B and is broadly distributed among

several tissues. It is present at elevated and constant levels in CTLs and APCs, as well as in many endothelial cells and at sites of immune privilege [187, 188], pointing to an immune modulatory function of this protein.

Induction of PI-9 expression is mainly triggered by IFN-γ and the Hypoxia Inducible Factor 2 (HIF-2), but also lipopolysaccharide (LPS) and interleukin-1β (IL-1β) in inflammation, as well as estrogens are known to contribute to PI-9 expression [189, 190]. In cells that express PI-9 and granzyme B (*e.g.* CTLs, NK-cells), intracellular serpins are thought to provide an important mechanism for the cell to evade death caused by the release of compartmentalized cytolytic proteases, that may leak into the cytosol during their storage in cytotoxic granula, or during the degranulation of the latter as the CTL executes its apoptotic functions [191].

There is evidence that ectopic PI-9 expression in tumor cell lines can protect against the cytolytic effects of granzyme B and perforin [192], and high levels of PI-9 are associated with a poor therapeutic response and prognosis in lymphomas and melanomas [193]. The association with hematopoietic tissue makes PI-9 an interesting candidate for evaluation of immune evasion in AML.

1.5 T cells and functional inhibition

T cell functions can be divided into three parts: activation, killing and regulation. Each of these processes can therefore be targeted to inhibit proper effector-functions of T cells, including constrained or lacking cytokine- or perforin-production or transformation of the T cell into an anergic state [194, 195]. These functions can be attributed to different subsets of T cells, which can be characetrized phenotypically: The classical approach uses the expression of the surface antigens CD4 and CD8 to segment $CD3^+$ cells in two major functional subpopulations:

T cells sharing the CD4 surface antigen are classified as helper T cells (mainly T_H1, T_H2 and T_H17) and if activated, proliferate and secrete cytokines that provide essential co-stimulatory signals for activation and differentiation of cytotoxic T cells (CTLs), B-cells and macrophages for a complete immune response.

CD8 is a common feature of CTLs which are responsible to lyse abnormal cells *e.g.* virally-infected, transformed or transplanted cells. The specific recognition via MHC-class-I/TCR interaction of a target cell by a CTL usually leads to induced apoptosis of the target cell, mostly via one of two mechanisms:

a.) Induction of apoptosis via cytotoxic granula, modified lysosomes, that contain cytotoxic effector proteins, as perforin, granzyme and granulysin, which release proapoptotic pathways in the target cell, or

b.) Through the activation of death-receptors, like FasR und TRAIL-R, subsequently inducing proapoptotic signal cascades. This mechanism, in contrast to perforin-mediated apoptosis, is mainly used to lyse pathogen-specific lymphocytes after a cured infection to limit further proliferation.

1.5.1 T cell subsets, functionality and homing

As most cells during their live cycle, T cells traverse different phases of forming and function. This developmental process is strongly associated with the kind of challenge the cell experienced and is accompied by phenotypic transformations.

The actual downstream process of T cell transformation is currently subject of a controversial discussion, implementing models of linear and divergent pathways of T cell differentiation [196-199]. However, the differential expression of four molecules, CD45RA, CCR7, CD27 and CD28, is widely accepted to distinguish between numerous subsets of "resting" (meaning cells that are not involved in any infection phase) T cells. According to this characterization, T cells are divided into naïve, effector(T_{EFF}), effector memory (T_{EM}), central memory (T_{CM}) and highly differentiated subpopulations [200]. After primary contact to an antigen, naïve T cells differentiate into effector cells, traveling to the site of infection or settings of active antigenic stimulation (*e.g.*, primary viral infection, vaccination), respectively. T_{EFF} are able to eliminate viruses or tumors by different effector functions as mentioned above. The effector phase of the response is short, with a rapid expansion of antigen-specific T cells and pathogen clearance. The expanded effector cells undergo a contraction phase, while approximately 5% to 10% of antigen-specific cells are maintained to establish a memory pool and provide long-term protection from a secondary challenge by the same pathogen.

The functional requirements mirror the phenotypic transformation: As the interactions of different subsets of immune and non-immune cells at defined sites are required for the efficient function of the immune system, the ability of these cells to actively migrate to and within tissues is regulated by the expression of different chemotactic receptors.

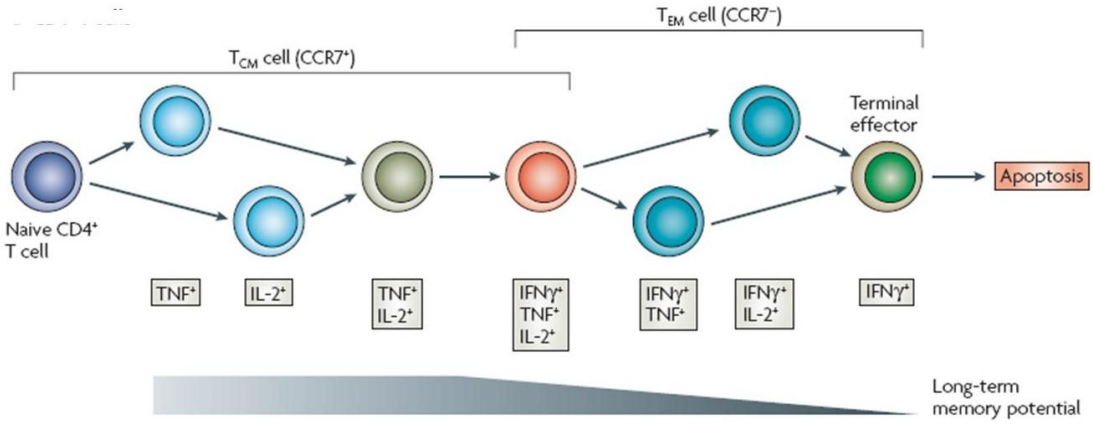


Figure 9: Maturation-process within the major memory T cell subsets in humans. T cell differentiation can be modelled as a linear process, in which cells progressively gain functionality with further differentiation, until they reach the stage that is optimized for their effector function (such as the production of different cytokines). Consecutive antigenic stimulation can lead to progressive loss of memory potential, which is accompied by reduced CCR7-expression as well as cytokine production, resulting in terminally differentiated T cells that only produce IFN-γ and are short-lived. Figure adapted and modified from Seder *et al.* [8]

One of these receptors, CC-chemokine receptor 7 (CCR7) is associated with the subpopulations' potentials to establish a long-term memory [8] (Figure 9) and is also essentially involved in homing of various subpopulations of T cells (naive T cells, T_{CM} cells and T_{regs}) as well as antigen-presenting dendritic cells (DCs) to the lymph nodes (Figure 10). For instance, naïve T cells, which are $CCR7^+$, migrate into secondary lymphoid organs (SLOs), including peripheral lymph nodes, Peyer's patches and the spleen to first encounter cognate antigen and become activated [9]. In contrast, human T_{EM} do not express CCR7 but display characteristic sets of chemokine receptors and adhesion molecules that are required for homing to sites of inflammation.

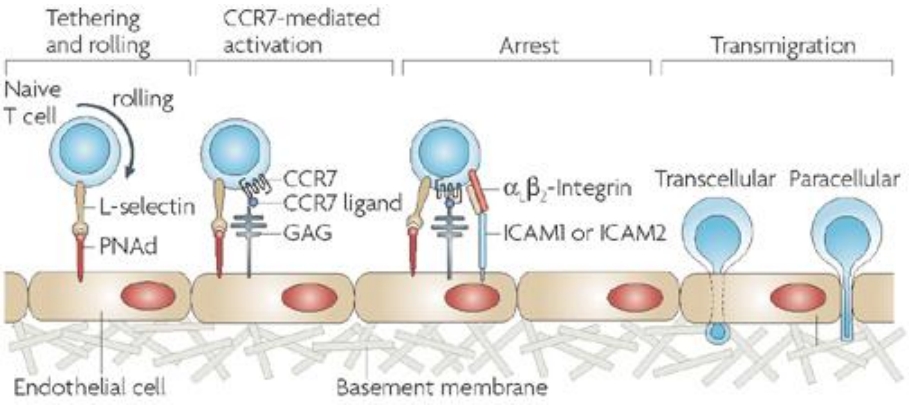


Figure 10: The multi-step process of homing to the lymphnodes. Homing T cells emigrate from the blood to peripheral lymph nodes in a multistep process, consisting of tethering and rolling, CCR7-mediated activation, firm arrest and transendothelial migration. Figure adapted from Förster *et al* [201].

In the human system, the antigen-experienced cells have been divided into central memory- and effector memory-subsets based on two distinct criteria: (*a*) the absence or presence of immediate effector function and (*b*) the expression of homing receptors that allow cells to migrate to secondary lymphoid organs versus non-lymphoid tissues [199]. T_{CM} express the lymph node homing receptors L-selectin and CCR7 and, like naive T cells, are well represented in all SLOs.

T_{CM} have higher sensitivity to antigenic stimulation, are less dependent on co-stimulation, and up-regulate CD40L to a greater extent, thus providing more effective stimulatory feedback to DCs and B cells than do naïve T cells. T_{CM} have little or no effector function, but are able to readily proliferate and differentiate to effector cell upon antigenic encounter. As indicated by the nomenclature, T_{EM} exhibit increased and rapid effector functions in terms of perforin loading and cytokine response to antigenic stimulation [202].

1.5.2 T cell exhaustion and anergy

Due to its high potency, the immune system has to be strictly regulated to avoid "collateral-damage" to unaffected cells and tissue. The concept of immune checkpoints, a series of factors that limit an ongoing immune response, has attracted considerable interest in cancer immunotherapy [6, 7]. According to this concept, the activity of peripheral T cells can be modified by positive and negative regulatory receptors leading to exhaustion or anergy if bound by the corresponding ligand. Multiple modulators are able to suppress exaggerated reactions of CTLs, a pathway usually used after pathogen-eradication. The most prominent of these regulators are part of the CD28/CTLA-4-family [5, 203]: CD28 and the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) are expressed on T cells to bind to B7 family members expressed on antigen presenting cells, but result in different reactions: While the ligation of CD28 to the corresponding receptors, B7-1 or B7-2, facilitates T cell activation and promotes T cell expansion and differentiation [204], the ligation of CTLA-4 to the same receptors results in inhibition of TCR and CD28 mediated signals[205] (Figure 11).

Several more members of the B7-CD28-family have been recently identified and can be correlated to the development or maintenance of immune tolerance. Recent investigations concerning this subject showed that multiple negative regulatory mechanisms appear within the tumor microenvironment to potently inhibit even

strongly primed antitumor T cell responses. The most common inhibitory mechanisms not provoked by the tumor, seem to be T cell anergy due to poor co-stimulation by tumor cells, suppression of conventional T cell function by regulatory T cells or other regulatory cell types, and engagement of Programmed Death-1 receptor on activated T cells with PD-L1.

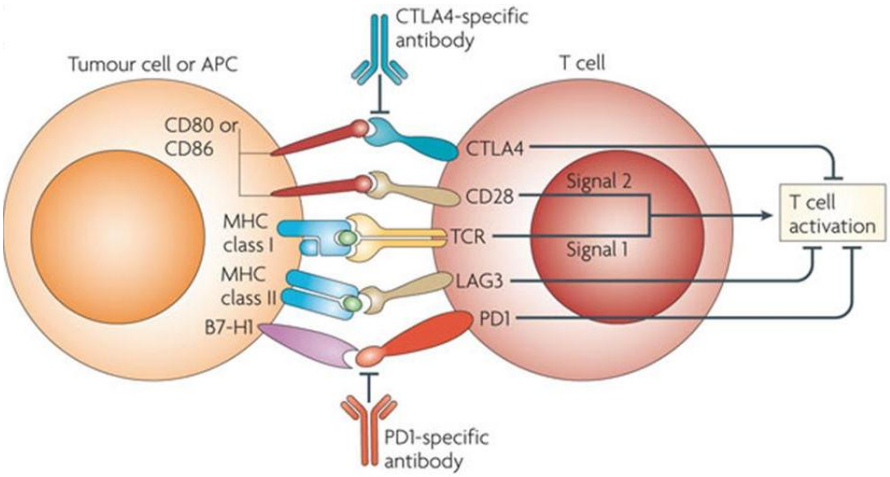


Figure 11: Influence of immune checkpoints on the proliferative capacity of T cells. Besides the recognition of the corresponding MHC/peptide complex, a T cell needs additional trigger like CD28 activation to initiate cytokine production and proliferation. Consecutive activation of the T cell induces the expression of negative regulators, *e.g.* PD-1, binding to B7-H1 and CTLA-4, competitively binding to the same ligands as CD28. Activation of these regulators initiates signals that have opposite effects to those of CD 28/TCR activation and restrict proliferation, thus driving the T cell in an anergic state. Current immune checkpoint therapies target these interactions by the administration of interfering antibodies, resulting in a blockade of immune suppression. Figure adapted from Charles G. Drake [206].

First identified as a gene up-regulated in a T cell hybridoma undergoing cell death ([203]), PD-1 is an immunoinhibitory receptor of the immunoglobulin (Ig) superfamily, sharing approximately 23% AA sequence homology with CTLA-4. PD-1 is expressed by chronically activated T cells, B cells, and myeloid cells [207]. A wider distribution of PD-1 than the predominantly T cell-expressed surface molecules CD28 and CTLA-4 suggests a more diversified role in immune regulation. The variety of immune diseases displayed by PD-1 knockout mice[208, 209], as well as the association of increased viremia in patients with chronic HIV[210, 211] and HCV[212] infections with high expression of PD-1 indicate a critical role of PD-1 in lymphocyte homeostasis.

The engagement of PD-1-with one of its ligands, PD-L1 or PD-L2, results in impaired T cell proliferation and reduced cytokine production [159]. In the context of tumor therapy, it is important to notice, that expression of PD-L1 is reported on

many entities of human tumors, including AML [213], as well as on tumor-associated DCs [214]. Interestingly, although a lot is known about the expression of PD-L1, only rudimentary information is available on the pattern of PD-1 expression in AML and its direct implications on phenotypic and functional characteristics on tumor infiltrating T lymphocytes (TILs).

1.6 Immune regulatory cells

1.6.1 Regulatory T cells

The third T cell function – regulation – can not be attributed to a distinct T cell compartment. So called regulatory T cells (T_{regs}) derive in most cases from the $CD4^+$ subpopulation, but $CD8^+$ T cells displaying regulatory functions have been described recently [12, 215].

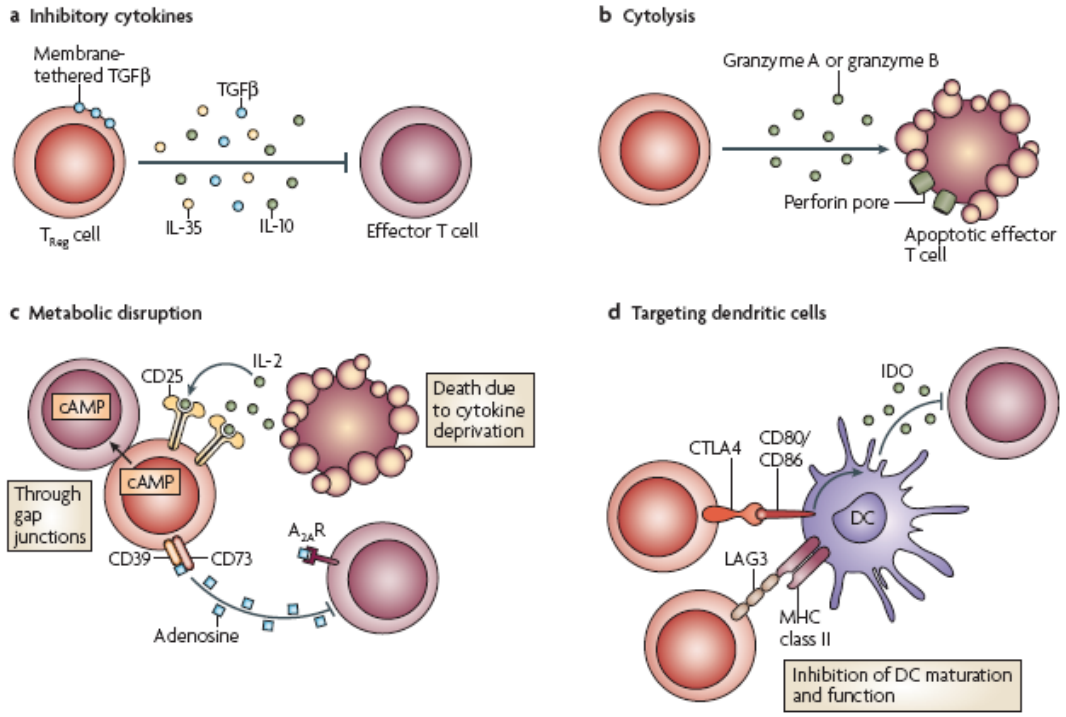


Figure 12: Basic suppressive mechanisms used by T_{reg} cells. a) Inhibitory cytokines **b)** Cytolysis **c)** Metabolic disruption **d)** Modulation of DC maturation and/or function. Adapted from D. A. A. Vignali *et al.* [11]

While T_{reg} activity is usually required for normal immune homeostasis, dysregulation of their numbers can induce autoimmunity or aid in the pathogenesis of disease, *e.g.* systemic lupus erythematosus, rheumatoid arthritis or psoriasis. Phenotypically, $CD25^+CD127^{dim}$- or $CD25^+FoxP3^+$- expression actually are the state of art to discriminate T_{regs} from classical T cells. Regulatory T cells do not proliferate in

response to antigen binding but prevent the activation of helper and cytotoxic T cells, *i.e.* they exhibit regulative functions to limit the extent of immune activation (Figure 12). T_{reg}-mediated immunosuppression has also emerged as a crucial mechanism of tumor evasion, which is thought to potently contribute to the observed lack of responses to immunotherapy in cancer patients [216, 217].

T_{reg} can suppress $CD4^+$ and $CD8^+$ T cell proliferation and the production of effector cytokines regardless of TCR specificity. [215, 218] This is mediated, at least in part, by inhibition of IL-2 transcription within the target cell. T_{regs} constitutively express CTLA-4[219] and secrete inhibitory TGF-beta and IL-10.[220, 221] T_{reg}-activity requires direct contact with the target T cell, although the molecular mechanism for suppression is not clear.

1.6.2 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a phenotypically heterogeneous cell population that includes mature myeloid cells, such as granulocytes, monocytes/macrophages, and DCs, as well as immature myeloid cells (IMCs).[222] Their presence has been observed in most cancer patients, where they are induced by various factors produced by tumor cells and/or by host cells in the tumor microenvironment, and are considered a major contributor to the profound immune dysfunction of most patients with sizable tumor burdens [222, 223]. Limited efficacy of current vaccination strategies is also thought to be partly attributed to MDSCs [10, 224].

Features that are common to all MDSCs are their myeloid origin, their immature state and their ability to suppress T cell responses by limiting T cell function to cell-surface receptors and/or through the release of soluble mediators with short half-lives. Therefore, the definition of MDSCs occurs rather on their biological activity than on their phenotype. Nevertheless, there have been phenotypic approaches to characterize this heterogeneous population: human MDSCs are usually defined as $CD14^-CD11b^+$ cells that express the myeloid marker CD33 but neither MHC-class-II, co-stimulatory molecules, or other mature myeloid and lymphoid markers [223].

In healthy individuals, IMCs constitute approximately 0.5% of peripheral blood mononuclear cells. In contrast to healthy individuals, where IMCs quickly differentiate into mature granulocytes, macrophages or dendritic cells, partial blocking of IMC maturation in pathological conditions (*e.g.* cancer, various

infectious diseases, sepsis and trauma) results in the expansion and accumulation of this population (Figure 13) [222].

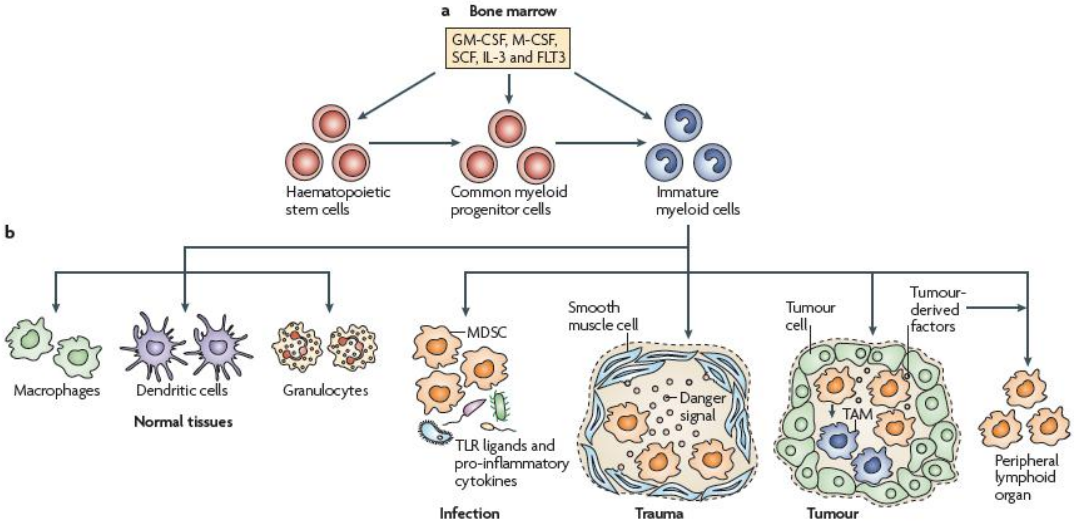


Figure 13: The origin of MDSCs. a) Immature myeloid cells (IMCs) are part of the normal process of myelopoiesis, controlled by a complex network of soluble factors, including cytokines and cell-expressed molecules. **b)** Normally, IMCs migrate to different peripheral organs, where they differentiate into macrophages, dendritic cells or granulocytes. Factors that are produced during acute or chronic infections, trauma or sepsis and in the tumor microenvironment promote the accumulation of IMCs, prevent their differentiation and induce their activation. Figure adapted from D. I. Gabrilovich *et al.* [222].

If activated, IMCs up-regulate the expression of immune suppressive factors, such as arginase 1 and nitric oxide synthase (NOS2), resulting in an increased production of nitric oxide (NO) and reactive oxygen species (ROS). The increased activity of arginase 1 in MDSCs leads to enhanced L-arginine catabolism, which depletes this non-essential amino acid from the microenvironment. The deprivation of L-arginine then inhibits T cell proliferation through several different mechanisms, mainly affecting the expression of CD3 and cell cycle regulators [225, 226].

NO suppresses T cell function through various different mechanisms that involve the inhibition of JAK3 and STAT5 functions in T cells [227], the inhibition of MHC class II expression and the induction of T cell apoptosis [138]. Generally, MDSC have been shown to contribute to the inhibition of NK cell cytotoxicity [228], the perturbation of lymphocyte trafficking [229] and the induction and accumulation of T_{reg} cells [230, 231]. Interestingly, a recent publication shows evidence, that there exists a co-dependent, or, as the authors entitle it, a retaliatory relationship between T cells and MDSC in that MDSC suppress T cell activation, but, once activated, T cells are able to mediate MDSC apoptosis via the Fas/FasL-pathway [232], providing a new and surprising perspective to an intricate suppressive mechanism.

1.7 Immune assays / Immunomonitoring

The detection and enumeration of disease-specific T cells and their antigenic specificity are of major importance in T cell immunology and its application in targeted therapy. However, the valid enumeration of spontaneous and therapy-induced T cell responses remains challenging, as generally low frequencies of tumor-specific T cells (range from $1x10^4$ to $1x10^5$) request sensitive, ccurate and reproducible detection methods. Especially in the context of immune monitoring, where the quantification and the assessment of functionality of specific T cells are routinely performed, standardized and accurate assays represent a major pre-requisite [233].

On this account, fluorescent peptide/MHC-multimers [234] are widely used to directly identify, quantificate and phenotype antigen-specific T cells by flow-cytometry. Their application in combination with *fluorescence-activated cell sorting* (FACS) and/or *magnetic-activated cell sorting* (MACS) even allows purification of antigen-specific T cells. Admittedly, peptide/MHC-multimer do not deliver information concerning effector functions of the targeted T cells, requiring additional functional or combined assays.

Current functional assays, including the *enzyme-linked immuno spot technique* (ELISPOT)[235] as well as intracellular-cytokine flow-cytometry (ICC), are both able to detect an antigen-induced cytokine response on the single-cell-basis.

The ELISPOT-assay represents a sensitive[236, 237] and reproducible[238-240] technique with a broad range of target molecules like IFN-γ, TNF-α, TGF-β, Granzyme B, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, GM-CSF, Fas-Ligand and MMP-1, which has been adapted for high-throughput processes [241]. However, it lacks information on the phenotypic composition of the tested samples, which, in contrast, represents a benfit when using the ICC-technique. Current flow-cytometers are able to detect up to 20 parameters, allowing defined and simultaneous characterizations on the phenotypic and functional analysis of the investigated T cells[242], covering multiple features as the memory/effector status, the proliferative capacity[243], the activational state, the cytokine profile as well as the cytolytic function[244]. In addition, ICC can be combined with peptide/MHC-multimer analyses. Yet, the increasing amount of available dyes exacerbates the optimal selection of fluorochrome-combinations as well increases interpretative variances between different cytometer-facilities [245]. On this account, current multi-center-initiatives to

harmonize ELISPOT-, ICC- and MHC-multimer-assays have been established to facilitate future data comparison [246-248].

Nevertheless and despite these convenient characterizational approaches, functional analyses can not be circumvented when analyzing T cell reactivity and immunity. Therfore, the functional and cytotoxic capacity of CTLs is usually determined by cytolytic assays.

Effector cell-mediated cytolysis is usually detected by alterations of plasma membrane permeability and integrity and the resulting release of components into the supernatant or the uptake of dyes that are normally excluded by viable cells. Assays focusing on the release of tracer reagents frequently used to contain radioactive isotopes, *e.g.* ^{51}Cr, ^{75}Se or ^{3}H. As the use of these products is potentially hazardous and includes special training, the detection of cell-mediated cytolysis in a more convenient way represents a certain requirement in scientific research. Therefore, several non-radioactive tracer assays have been developed, but the indirect results often limit the accuracy of these measurements, especially for antigens with low immunogenicity [249]. Additionally, with growing knowledge regarding apoptosis, the disadvantages of these assays grew as well. For instance, early phases of apoptosis don't affect membrane permeability. Altenative assays therefore focus on structural changes occurring during those stages. One compound that translocates from the inner to the outer leaflet of the plasma membrane is the lipid phosphatydilserine (PS), which can easily be detected by extracellular staining with fluorochrome-coupled AnnexinV[250]. In combination with the DNA-intercalator propidium-iodide (PI), dead cells can then easily be separated from apoptotic cells. Nevertheless, the use of PI also requires carefull handling and excessive rinsing of the used ctometers. Actually, several innovative fluorescent dyes have been introduced and are now widely used [251, 252] among the scientific community.

2 Objectives

The capacity of T cells to induce an immune response, against foreign pathogens or cancerous cells represents the basis of immunity. Successful cancer immunotherapy therefore relies on the quantitative as well qualitative effector function of pathogen-specific T cells. Nevertheless, the tumor microenvironments as well as the tumor cells themselves are able to evade immunosurveillance and/or to impair proper T cell responses. It seems evident, that a loss of function of the host immune system is an important factor limiting the success of cancer immunotherapy.

The foregoing study investigated the immunogenicity of Wilms' tumor gene product 1 (WT1)-peptide vaccination in WT1-expressing acute myeloid leukemia (AML) patients without curative treatment option. Despite the first immunologic, molecular, and preliminary evidence of potential clinical efficacy in AML patients, only in a few cases long-lasting responses could be documented. It is known that enduring efficacy of cancer vaccines may be limited due to immune escape mechanisms.

As analyses for loss or mutations of the WT1 epitope or epitope flanking sequences that may impair proper T cell recognition or epitope presentation turned out negative, this work was initiated to reveal further potential immune escape mechanisms. We chose to work on three front lines: Investigations of immune modulatory counter-attack-mechanisms of the tumor, functional deficiencies of the T cell compartment and the presence of immune regulatory cells (summarized in Figure 14).

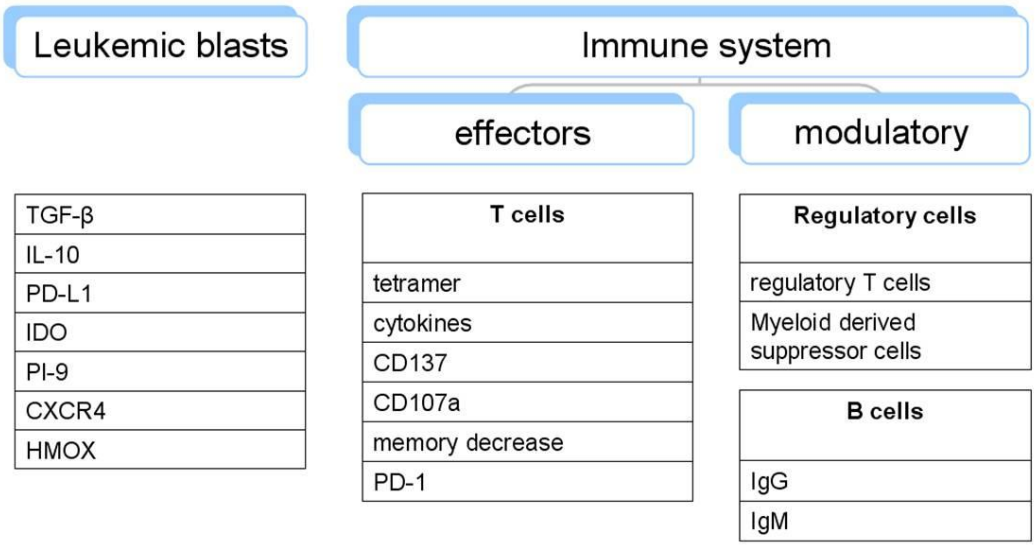


Figure 14: Outline of the targeted factors in this work which may interfere wit vaccine efficacy.

3 Material and Methods

3.1 Patients

3.1.1 Patients characteristics and vaccination regimen

The analyses were performed in a subgroup of patients with relapsed AML, first remission AML at high risk of relapse, or untreated AML, who were included in a phase II vaccination study with WT1 peptide, KLH and GM-CSF[93].

Patient	Age	FAB	Karyotype	Vacc.	Clinical response	PFS
1	66	M4	Normal	12	CR 17 months from week 8 on	514
2	67	M2	46 XX; t 11,q23	12	Persistent CR 36+ month	978
3	82	M2	Normal	4	SD 3 months	136
5	72	M2	Normal	6	PD after # 2	37
6	74	M1	47 XX +11/46 XX (23)	9	SD for 4 month	201
7	67	M2	46 XY/47XY,+8	9	Relapse after 4 month	128
8	45	M7	Normal	4	PD after # 4	50
9	68	M5B	Normal	12	Relapse after 10 month	310
10	73	sAML	Normal	12	SD and erythr. resp. for 7 months	262
11	63	sAML	Normal	18	SD for 4 month	101
12	80	M4	Normal	8	SD for 3 month from week 10	144
13	20	M4	Normal	5	Relapse after 2 month	68
14	59	M4	Normal	4	PD after #4	29
15	32	M2	47XY+; t3;21	5	Relapse after #5	57
16	65	M4	Normal	12	SD for 2 months/PD after week 18	134
17	67	RAEB II	Normal	24	SD 10 months	339
18	61	sAML	Normal	11	SD 5 months	142
19	69	RAEB II	Normal	10	SD for 2 months	69
20	75	sAML	Comple X	10	SD for 2 months	132
21	82	M4	Normal	11	SD for 4 months	149
22	65	M2	Normal	15+	Ongoing remission since	1682
23	58	M2	Normal	14+	SD ongoing since	1668
24	72	sAML	Normal	13+	Initial PD, SD 2 months	504
25	73	sAML	Normal	11	Relapse after 5 months	147
26		sAML	46XX (del5)	11+	SD for 4 months	99

Table 6: Patients characteristics and clinical response.

Vaccines were injected intradermally and subcutaneously, approximately 10 cm distal of the right groin. One vaccination comprised four days of 62.5 µg GM-CSF (Leukine®, Berlex, Seattle) and on day three 0.2 mg of the HLA-A201-restricted WT1.126-134 epitope peptide RMFPNAPYL (Clinalfa, Switzerland) admixed with 1mg KLH (Immucothel®, biosyn, Germany).This clinical trial had been approved by the Institutional Ethics Committee, and written informed consent was obtained from all patients and healthy subjects before sampling.

3.1.2 Sample collection

Sera and heparinized peripheral blood samples were obtained from each patient at different time points. Serum was frozen at -20°C and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Hypaque and cryopreserved.

3.2 Healthy controls

Buffy coats were obtained from blood donors from DRK. PBMCs were isolated using a density-gradient (Ficoll Hypaque) and cryopreserved. Lymphocytes were HLA-typed and tested for reactivity towards Influenza A, CMV and EBV.

3.3 Human cell lines

line	ATCC-number	DMSZ-number	Disease	Cell type	Reference
HL-60	CCL-240	ACC 3	AML	promyeloblast	[253]
K-562-A2*	CCL-243	ACC 10	CML	erythroblast	[254]
Molt-4	CRL-1582	ACC 362	ALL	T lymphoblast	[255]
TF-1	CRL-2003	ACC 334	erythroleukemia	erythroblast	[256]
THP-1	TIB-202	ACC 16	AML	monocyte	[257]
UT-7	n.a.	ACC 137	AML	megakaryocyte	[258]

Table 7: Summary of available and used human leukemic cell lines. The listed cell lines were purchased via the American Type Culture Collection (ATCC) or the *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH* (DMSZ, German Collection of Microorganisms and Cell Cultures). * K562-A2 is an HLA-A2 transfected cell line based on K562. The provided information in this table therefore applies to the original cell line.

3.4 Antibodies and staining reagents

Antibody	Conjugate	Manufacturer	Antibody	Conjugate	Manufacturer
CCR7	-	BD	CD86	FITC	BD
CD107a	PE	BD	GAM	Biotin	Southern biotech
CD11b	PE-Cy7	BD	GAM	PE	BD
CD127	FITC	eBioscience	HLA-A2	Alexa647	AbD Serotec
CD137	APC	BD	HLA-DR	PE	BD
CD14	PerCP	BD	HO-1	FITC	Abcam
CD14	APC	BD	IFNy	FITC	BD
CD19	FITC	Coulter Biotech	IgG	Peroxidase	Nordic Laboratories
CD25	PE	BD	IgM	Peroxidase	Nordic Laboratories
CD274	APC	BD	Il-2	APC	BD
CD3	AmCyan	BD	Il-2	Biotin	BD
CD3	FITC	Coulter Biotech	INDO	PE	USbiological
CD3	PerCP	BD	PD1	PE	BD
CD33	PE-Cy7	BD	PI-9	APC-H7	Santa Cruz
CD34	PE-Cy7	BD	SAV	PerCP	BD
CD4	PE-Cy7	BD	SAV	750	Invitrogen
CD56	FITC	BD	TNFa	PE-Cy7	eBioscience
CD8	APC-H7	BD	ViVid	PacificBlue	Invitrogen
CD80	FITC	BD	WT1	-	Dako

Table 8: Register of antibodies and the corresponding distributors.

3.5 ELISA to detect anti-KLH immunoglobulins

For the detection of KLH-specific antibodies (IgM- and IgG-subtypes), 96-well plates were coated with KLH (50µg) for 1h and washed twice with *aqua dest.* Blocking was performed with BSA for 30 min followed by two washing steps. Plates were then incubated with sera (diluted 1:10 in PBS) for 30 min and washed twice again. Consecutively, peroxidase (PO) -conjugated anti-IgM or anti-IgG antibodies (Nordic Immunological Laboratories, Eindhoven, Netherlands) were allowed to bind KLH-specific Igs for 30 min. After two washing steps, 100 µl of the peroxidase substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS)[259] (Millipore, Schwalbach/Ts., Germany) was allowed to react with remaining PO-conjugates. Extinction was measured at $\lambda = 550$ nm. Positivity was determined as the mean value of three healthy donors increased by the triple standard deviation. As positive control, a serum mix of orally with KLH immunogenized patients was kindly provided by J. Maul.

3.6 Western-Blot to detect anti-hGM-CSF immunoglobulins

Sera of patients were tested for anti-GM-CSF antibodies (isoformes IgG and IgM) via Western-Blot as previously described [260]. Briefly, GM-CSF (Leukine; Berlex, Seattle, WA, USA) was run on a 15% SDS-PAGE and transferred to Hybond-C nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). After stripping, the membrane was blocked overnight with skimmed milk (5% (Milupa, Friedrichsdorf/Ts., Germany) in PBS with 0.01% Tween-20) and incubated with serum for 1 h, washed twice with PBS-T and probed with Peroxidase-conjugated antibodies to human Igs (Nordic Immunological Laboratories). Development was performed with a DAB-System (Vector Laboratories).

3.7 MTT-Assay

For the detection of neutralizing antibodies to GM-CSF we performed a MTT-Assay (Cell Proliferation Kit I (MTT), Roche, Basel, Switzerland). Culture medium containing hGM-CSF (0.5 ng/ml) was preincubated with patients' sera in 96 well, flat bottom microplates. TF-1 cells were added at a concentration of 5×10^3 cells/well in 50 µl culture medium and incubated for 48 h in a humidified atmosphere (37°C, 5% CO_2). After the incubation period, 10 µl of the MTT labeling reagent (final concentration 0.5 mg/ml) were added to each well. The microplate was then incubated for 4 h, before 100 µl of the solubilization solution was put into each well. After overnight incubation the plate was checked for complete solubilization of the purple formazan crystals and photospectrometrically measured at 650 nm using an ELISA reader.

3.8 Cell culture

3.8.1 Passaging of human cell lines

Human leukemic cell lines were cultured in RPMI medium (PAA, Linz, Austria) supplied with FCS (10%, PAA). Depending on the cell line and according to the references, specific adjuvants as additional FCS, GM-CSF or gentamycin were added. The cell lines were initially seeded in a 6-well plate (Sarstedt, Nümbrecht, Germany) and transferred into a 100 ml culture flask (BD Falcon, Franklin Lakes, NJ USA) after 2 days. Culture medium was then consecutively refreshed every 2-3 days.

3.8.2 Expansion of antigen-specific T cells

Protocol 1

PBMCs were thawed and incubated in Iscoves medium (PAA) supplied with human AB serum (10%, PAA). Mononuclear cells from PB samples were cultured together with different peptides, IL-2 (50 IU/ml; R&D Systems, Wiesbaden, Germany) and IL-7 (10 ng/ml; R&D Systems) for 10 days for initial antigen-priming. During that period medium and leukines were refreshed every 2-3 days.

Protocol 2

PBMCs were thawed and incubated in Iscoves medium (PAA) supplied with human AB serum (10%, PAA). Mononuclear cells from PB samples were cultured together with different peptides, IL-4 (5 ng/ml; R&D Systems) and IL-7 (10 ng/ml; R&D Systems) for 10 days for initial antigen-priming. During that period medium was refreshed every 2-3 days. The medim was inoculated with IL-2 (50 IU/ml) and IL-4 (5 ng/ml) on a two-day interval.

Protocol 3

PBMCs were thawed and stained with fluorochrome-conjugated antibodies to CD3 (FITC), CD8 (PE) and with fluorochrome-conjugated $WT1_{128-134}$-Tetramers (APC). CD3, CD8 and $WT1_{128-134}$-Tetramer triple positives were sorted as single cells into 96 well plates, containing approx. 10^5 feeder-cells per well together with phytohemagglutinin (PHA) and 120 U/ml IL-2. Plates were incubated and medium was changed every 3-4 days. Following 10 – 14 days of incubation, growing clones were transferred into new 96 well plates together with feeder cells and further expanded.

Protocol 4

PBMCs were thawed and incubated in Iscoves medium (PAA) supplied with human AB serum (10%, PAA). Mononuclear cells from PB samples were primed with different peptides. On day 1 IL-7 (20 ng/ml) and IL-12 (0.5 ng/ml, R&D Systems) were added and the cells were incubated for 10 days. During that period medium was refreshed every 2-3 days and inoculated with IL-2 (30

IU/ml). In addition, the expanded cells were weekly restimulated with autologous irradiated and peptide-primed PBMCs.

Protocol 5

PBMCs were thawed and incubated in Iscoves medium (PAA) supplied with human AB serum (10%, PAA). Mononuclear cells from PB samples were primed with different peptides. On day 1 IL-7 (20 ng/ml), IL-10 (10 ng/ml, R&D Systems) and IL-12 (0.1 ng/ml) were added and the cells were incubated for 10 days. During that period medium was refreshed every 2-3 days and inoculated with IL-2 (30 IU/ml). In addition, the expanded cells were weekly restimulated with autologous irradiated and peptide-primed PBMCs.

Protocol 6

PBMCs were thawed and incubated in Iscoves medium (PAA) supplied with human AB serum (10%, PAA). Mononuclear cells from PB samples were primed with different peptides. On day 1 IL-6 (10 ng/ml, R&D Systems) and IL-12 (5 ng/ml) were added and the cells were incubated for 10 days. During that period medium was refreshed every 2-3 days and inoculated with IL-2 (30 IU/ml). In addition, the expanded cells were weekly restimulated with autologous irradiated and peptide-primed PBMCs.

Protocol 7

PBMCs were thawed and incubated in Iscoves medium (PAA) supplied with human AB serum (10%, PAA). Mononuclear cells from PB samples were primed with different peptides. On day 1 IL-7 (20 ng/ml) and on day 5 IL-15 (5 ng/ml, R&D Systems) were added and the cells were incubated for 10 days. During that period medium was refreshed every 2-3 days and inoculated with IL-2 (30 IU/ml). In addition, the expanded cells were weekly restimulated with autologous irradiated and peptide-primed PBMCs.

Protocol 8

PBMCs were thawed and incubated in Iscoves medium (PAA) supplied with human AB serum (10%, PAA). Mononuclear cells from PB samples were primed with different peptides. On day 1 IL-6 (10 ng/ml) and IL-12 (5 ng/ml) were added and the cells were incubated for 10 days. During that period

medium was refreshed every 2-3 days and inoculated with IL-2 (30 IU/ml). In addition, the expanded cells were weekly restimulated with autologous irradiated and peptide-primed PBMCs and inoculated with IL-7 (10ng/ml).

3.9 Flow cytometric analysis

3.9.1 T cell panel

Following the thawing of cryopreserved samples and overnight resting, antigen-specific T cells were analyzed by intracellular cytokine staining after a short time of incubation with WT1 candidate epitopes or irrelevant peptides as described previously [261]. In brief, PBMC (2×10^6) were incubated with 10 µg/ml of each peptide and xxx of Monensin. After 2 hours, 10 µg Brefeldin A (Sigma, Deisenhofen, Germany) were added, and after 16 additional hours, PBMCs were stained with fluorochrome-conjugated monoclonal antibodies against surface molecules. Afterwards, *FACS Lysing Solution* and *FACS Permeabilization Solution* (Becton Dickinson) were added, and intracellular molecules were stained identically. Data acquisition was done on a FACSCantoII cytometer and analyzed using FlowJo Software. A T cell response was considered positive if the frequency of $CD3^+CD8^+$ T cells producing cytokines in response to the candidate epitope was at least 2-fold higher compared to an irrelevant peptide, and if there was a minimum of 0.05% of $cytokine^+CD3^+CD8^+$ T cells after the subtraction of the irrelevant peptide background response.

Antibody	Fluorochrome	Amount [µl]
CD3	AmCyan	5
TNFa	PE-Cy7	0.5
CD137	APC	20
IFNy	FITC	20
PD1	PE	15
Il-2	Biotin+SAV –	0.5+0.5
ViVid	PacificBlue	0.5
CD8	APC-H7	5

Antibody	Fluorochrome	Amount [µl]
CD3	AmCyan	5
TNFa	PE-Cy7	0.5
Il-2	APC	1
IFNy	FITC	20
CD107a	PE	20
CCR7	+GAM-Biotin+SAV	1+1+1
ViVid	PacificBlue	0.5
CD8	APC-H7	5

Table 9: Index of the two different staining panels used to identify possible T cell defects. Left panel: Focus on the exhaustion (PD-1) and/or impaired activation (CD137) of T cells. **Right panel:** Focus on impaired cytotoxic activity (CD107a) and phenotype (CCR7) of T cells.

3.9.2 T_{reg}-panel

Following thawing of cryopreserved samples and overnight resting, regulatory T cells were detected by FACS after an overnight-incubation with *Staphylococcus aureus* enterotoxin B (SEB) for CD137-detection.
PBMCs were then stained with fluorochrome-conjugated monoclonal antibodies against surface molecules as listed in Table 10 and analyzed using a FACSCantoII cytometer and FlowJo Software.

Antibody	Fluorochrome	Amount [µl]
CD4	PE-Cy7	3
CD8	APC-H7	5
CD137	APC	20
CD127	FITC	20
CD25	PE	10
CD3	PerCP	10
ViVid	PacificBlue	0.5

Table 10: Index of the antibodies used to detect regulatory T cells in PBMCs.

3.9.3 MDSC-panel

Following thawing of cryopreserved samples, MDSC were detected by FACS. PBMCs were stained with fluorochrome-conjugated monoclonal antibodies against surface molecules according to Table 11 and analyzed using a FACSCantoII cytometer and FlowJo Software.

Antibody		Fluorochrome	Amount [µl]
Trash :	CD19	FITC	10
	CD56		10
	CD3		10
CD11b		PE-Cy7	1
HLA-DR		PE	20
ViVid		PacificBlue	0.5
CD14		APC	3

Table 11: Index of the antibodies used to detect myeloid-derived suppressor cells in PBMCs.

3.9.4 Leukemic blast: immune regulatory-panel

Following thawing of cryopreserved samples and overnight resting, *FACS Lysing Solution* and *FACS Permeabilization Solution* were added, and extra- and intracellular molecules were stained as listed in Table 12 and analyzed using a FACSCantoII cytometer and FlowJo Software.

Antibody	Fluorochrome	Amount [µl]
CD3	AmCyan	5
CD33/34	PE-Cy7	4
CD274	APC	10
HO-1	FITC	0.5
INDO	PE	8
CD14	PerCP	5
ViVid	PacificBlue	0.5
PI-9	+GAM-Biotin + SAV750	8+0.5+0.5

Table 12: Index of the antibodies used to detect immune modulators on leukemic blasts.

3.9.5 Leukemic blast: WT1/HLA-A2-panel

Following thawing of cryopreserved samples and overnight resting, cells were fixed with 1% formaldehyde and permeabilized with 0.2% TritonX for 5 min on ice. For intranuclear staining, murine anti-hWT1 antibody was allowed to bind for 20 min. After consecutive incubation with goat-anti-mouse-PE antibody, additional extra- and intracellular molecules were stained time-delayed, due to their murine nature, as listed in Table 13 and analyzed using a FACSCantoII cytometer and FlowJo Software.

Antibody		Fluorochrome	Amount [µl]
CD80/86		FITC	15
HLA-A2		Alexa647	8
WT1		PE	3+GAM-PE ,5+
ViVid		PB	0.5
Trash:	CD3	PerCP	10
	CD14	PerCP	10

Table 13: Index of the antibodies used to determine WT1- and HLA-A2- expression on leukemic blasts.

3.10 Expression patterns of immune modulators by leukemic blasts

3.10.1 Control groups

To assess comparative data on the expression levels of the selected immune modulators, blood samples of healthy controls (n=5), of patients with different solid tumor entities (n=14) and of AML patients not included in our therapeutic setting (n=5) served as control-groups (Table 14).

Group	Patient	Sex	Born	Diagnosis
Healthy	M15	F	1977	
	M17	F	1967	
	M18	F	n. a.	
	M19	F	1979	
	M20	M	1979	
AML	P40	M	1929	AML
	P41	M	1928	AML
	P51	F	1927	AML
	P69	F	1966	AML
	P88	M	1935	AML
Entities	E1	M	1961	B-NHL
	E2	M	1940	Bronchial carcinoma
	E3	F	1935	Bronchial carcinoma
	E4	F	1940	Bronchial carcinoma
	E5	M	1942	Colorectal carcinoma
	E6	F	1937	Colorectal carcinoma
	E7	F	1933	Endometric / cervical carcinoma
	E8	M	1944	Hypopharyngal carcinoma
	E9	M	1949	Head/neck cancer
	E10	M	1947	Liposarcoma
	E11	F	1946	Oral cavity carcinoma
	E12	M	1928	RCC
	E13	M	1929	RCC
	E14	F	1945	Pankreatic carcinoma

Table 14: Characteristics of the control groups for qRT-PCR.

3.10.2 Messenger-RNA (mRNA) isolation

PBMCs were centrifuged at 1600 × *g* for 10 min and the cell pellet was resuspended in 1 ml of guanidium thiocyanate (GTC) buffer and stored at −80°C. Total RNA was isolated from PBMCs with the *RNeasy® Mini Kit* including *RNase-Free DNase Set* (both: Quiagen, Hilden, Nordrhein-Westfalen, Germany) according to the manufacturer's recommendations. Briefly, cells, stored at -20°C in GTC-buffer, were carefully thawed and applied on a *QIAshredder*-column and centrifuged. The filtrate was mixed (1:1) with 70% ethanol and transferred to an *RNeasy*-column. Intercepted by several centrifugation- and washing-steps, the sample was incubated with DNase and finally eluted in 53 µl of PCR-grade, RNase-free water. Determination of the

RNA-yield was performed on a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). RNA was stored at -80°C.

3.10.3 Complementary-DNA (cDNA) synthesis

Complementary-DNA-synthesis was performed with the *Omniscript® RT Kit* (Quiagen) according to the manufacturer's recommendations. Briefly, a maximum of 2 µg of RNA were diluted in 15 µl of RNase-free water, incubated for 5 min at 65°C, and placed on ice. A 7.5-µl mixture containing 2 µl of oligo-p$(dT)_{15}$ primer (0.8 µg/µl), 2 µl of dNTPs (5 mM), 0.5 µl of RNase-inhibitor (40 units/µl), 1 µl of *Omniscript®* Reverse Transcriptase (4.5 units/µl) and 2 µl of reverse transcriptase buffer (×10) was prepared and added to the diluted RNA. After incubation at 37°C for 1 h, *Omniscript®* Reverse Transcriptase was inactivated for 5 min at 95°C, and cDNA was stored at −20°C. All reverse transcriptase reagents, except oligo-p$(dT)_{15}$ primers and RNase-inhibitor (Roche Diagnostics, Mannheim, Germany), were purchased from Qiagen.

3.10.4 Quantitative real-time PCR

PCRs were performed on a LightCycler (Roche Diagnostics). Each cDNA (2 µl) was diluted to a volume of 20 µl of PCR mix (LightCycler Faststart DNA Master Hybridization Probes; Roche Diagnostics), containing 0.5 pmol of primer and 0.2 pmol of probe and a final $MgCl_2$ concentration as listed in Table 4.

	PD-L1	TGF	PI-9	HO-1	IL10	CXCR4	INDO
Annealing	57°C	57°C	58°C	58°C	60°C	56°c	60°C
Elongation	12 s	10s	10 s	10 s	10s	7s	12 s
[$MgCl_2$]	2,5 mM	5 mM	3 mM	3 mM	4 mM	4mM	3 mM

Table 15: Optimized qRT-PCR conditions for each of the immune modulators. Annealing temperatures, elongation time and $MgCl_2$-concentrations were determined by preliminary experiments.

Primers were purchased from Metabion (Martinsried, Germany) and fluorophore probes from TIB Molbiol (Berlin, Germany) and Metabion. For amplification, an initial denaturation step at 95°C for 10 min was used. For 50 subsequent cycles, the conditions were denaturation for 10 s at 95°C, annealing for 12 s at a target-specific temperature, followed by a target-specific elongation at 72°C (specific values provided in Table 15). Final extension was performed at 72°C for 2 min. The expected size of the PCR product was confirmed by agarose gel electrophoresis.All

samples were analyzed in duplicate on the expression of the control gene porphobilinogen deaminase (*PBGD*) and the target genes. The average value of both duplicates was used as a quantitative value. If only one of the duplicates gave a positive result, the positive result was taken.

3.10.5 Primers

Target	Type	Sequence
PD-L1	Forward	5'-ACT TAA AAG GCC CAA GC-3'
	Reverse	5'-CCG TTC CAA CAC TGA G-3'
	LC	5'-LC_{Red640}-GGA GTC AAA CAG GGA GCC T-Pho-3'
	FL	5'-AGC AGA GGA GGA GAA TGA AGA AAG-Fluo-3'
TGF-β	Forward	5'-CCC ACA ACG AAA TCT ATG AC-3'
	Reverse	5'-GCT AAG GCG AAA GCC C-3'
	LC	5'-LC_{Red640}-AGA GTG GTT ATC TTT TGA TGT CAC CG-Pho-3'
	FL	5'-GGC ACC CAG CGA CTC G-Fluo-3'
PI-9	Forward	5'-GTG CTT CGG CAT TTG G-3'
	Reverse	5'-CCC TTT ATG GCG ATG AG-3'
	LC	5'-LC_{Red640}-GTG TCT GTC CAA GTT CGT GC-Pho-3'
	FL	5'-GCA ATG TCA GCG GAG AGA GAC-Fluo-3'
HO-1	Forward	5'-CCC AAC GAA AAG CAC AT-3'
	Reverse	5'-CAG TGC CGT TAA ACA-3'
	LC	5'-LC_{Red640}-GTC AGC CCT GCC CTT CAG-Pho-3'
	FL	5'-AAA CTT CAG AGG GGG CGA AG-Fluo-3'
CXCR4	Forward	5'-GTG ACC GCT TCT ACC C-3'
	Reverse	5'-AGT CGA TGC TGA TCC C-3'
	LC	5'-LC_{Red640}-ATC CTG GCT TTC TTC GCC T-Pho-3'
	FL	5'-CCC TCA AGA CCA CAG TCA TCC-Fluo-3'
IL-10	Forward	5'-CCT TCC AGT GTC TCG G-3'
	Reverse	5'-TGG AGT ACA GGG GCA T-3'
	LC	5'-LC_{Red640}-AGG TCA GGA GTT CCT AAC CAG-Pho-3'
	FL	5'-AGG CGG GTG GAT CAC T-Fluo-3'
INDO	Forward	5'-ACG ATC ATG TGA ACC CA-3'
	Reverse	5'-AGC TCC TCA GGG AGA C-3'
	LC	5'-LC_{Red640}-CAG TCC GTG AGT TTG TCC TT-Pho-3'
	FL	5'-CTT CCT GTG CTC ATT AGA GTC AAA TCC-Fluo-3'
PBGD	Forward	5'-TGC AGG CTA CCA TCC ATG TCC CTG C-3'
	Reverse	5'-AGC TGC CGT GCA ACA TCC AGG ATG T-3'
	LC	5'-LC_{Red640}-TGT GGG TCA TCC TCA GGG CCA TCT TC-Pho-3'
	FL	5'-CGT GGA ATG TTA CGA GCA GTG ATG CCT ACC-Fluo-3'

Table 16: Index of primers utilized for qRT-PCR. LC = LC Red 640; FL = Fluorescein; Pho = Phosphate

3.10.6 Plasmid Controls, Standard Curve.

PCR products generated from cDNAs were cloned into the vector pCR2.1-topoisomerase (Invitrogen, Karlsruhe, Germany). Recombinant vectors, linearized with *Eco*RV, were serially diluted in water containing 0.4 µg/µl polyadenylic acid (Pharmacia Biotech, Freiburg, Germany). A standard curve with three dilutions of the respective plasmid in duplicates (1, 0.1, and 0.001 pg/µl) was included in each PCR run (Figure 15).

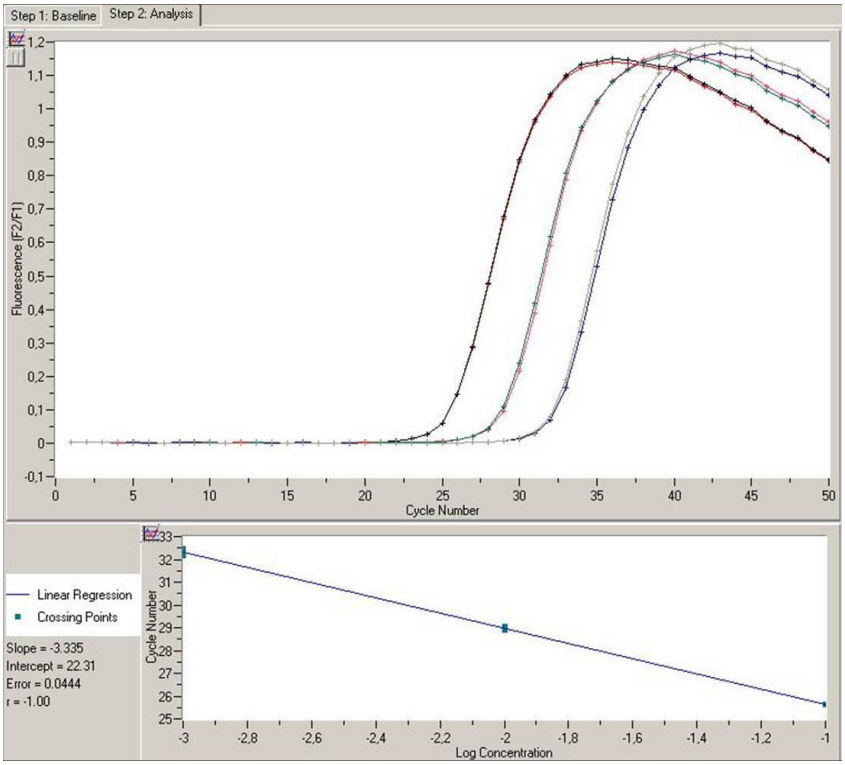


Figure 15: Standard curve and linear regression of the INDO-control. Indo-plasmid was amplified by PCR at different start-dilutions (1, 0.1, and 0.001 pg/µl). C_V-values were plotted versus $\log_{Concentration}$ and fitted by a trendline.

3.10.7 Precautions

To reduce risk of contamination, RNA extraction, cDNA synthesis, thermocycling, and post-PCR steps were performed in separate laboratories. PCR mixtures were set up in a template tamer (Oncor Appligene, Heidelberg, Germany). All reagents for cDNA synthesis were prepared with RNase-free water. For all RT-PCR steps, negative controls were performed as outlined in the EORTC recommendations[262], including a reverse transcriptase-negative sample control for every sample and water control for every PCR run.

3.10.8 Data Analysis

With the LightCycler software (version 3), crossing points were assessed and plotted *versus* the concentrations of the standards. The relative sample amount was expressed as ratio marker (*target gene*)[pg/µl]/(*PBGD*)[pg/µl]. The crossing points (beginning of the PCR exponential phase) for each reaction were determined by the Second Derivative Maximum algorithm, and sample concentration was calculated using the plasmid standard curve.

3.11 Cytotoxicity Assays

The methodical background of the cytotoxicity assays is explained in detail in section 4.7.

3.12 Stochastic evaluation

Stochastic evaluation concerning the correlation of the clinical response and the induction of WT1-specific IgM-antibodies was performed by using the standard t-test. Differences between healthy donors and AML patients were evaluated using the Mann-Whitney test, whereas variations between pre- and postvaccine samples were analyzed using the Wilcoxon matched pairs test. The statistical evaluation of the Kaplan-Meier curves, concerning PFS in terms of PD-1 expression and CCR7-variances were performed using the logrank test. Further statistical analyses for other functional assays were performed using the Mann-Whitney test.

4 Results

4.1 Humoral immunity against WT1 and KLH in AML patients vaccinated with WT-1-peptide plus GM-CSF and KLH

It has been shown that immunoglobulins specific to WT1 are present in patients with hematologic malignancies, comprising AML [124]. In the setting of a phase II vaccination we examined if WT-specific antibodies can be induced and/or boosted by the consecutive vaccination with an HLA-A201-restricted $WT1_{126–134}$ epitope in combination with KLH and hGM-CSF used as adjuvants. In 14 patients serum samples at baseline and during the course of vaccination were collected and screened for specific immunoglobulins.

4.1.1 Vaccination of leukemia patients with a MHC class I peptide of WT1 with unspecific T helper stimulation is able to induce WT1-specific IgM responses but fails to induce IgG responses

A

Patient	IgM				IgG			
	pre	W10	W18	W26/30	pre	W10	W18	W26/30
13	282	40			3343	2978		
14	100	41			3391	2260		
15	32	334			547	321		
16	419		1572		200		152	
17	93			1701	133			268
18	996	361	1634		101	97	57	
19	86	4			0	18		
20	9		122		1814		2213	
21	472		2424		283		194	
22	149	24	9	2603	0	24	53	0
23	21	167		1617	242	706		579
24	119	40	19	3561	69	24	54	487
25	140	7	456	1502	58	63	0	0
26	1950	745	236	1100	298	130	4	451

B

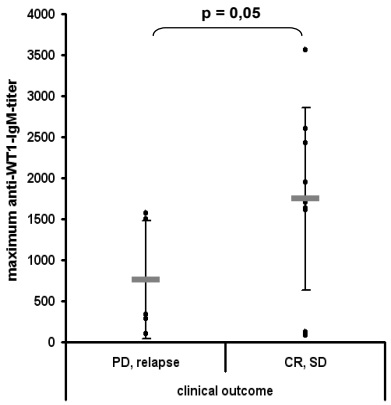


Figure 16: WT1-specific antibody profiles in PB and correlation of IgM induction and clinical outcome. A) Immunoglobulin-distribution during vaccination course. Values ≥ 700 for IgM and values ≥ 1000 for IgG were considered positive (mean of healthy donors + 2 fold SD). Each value shown represents an average of at least 2 experiments. B Correlation of IgM-titer and clinical outcome of patients. Mean values are indicated as grey bars. CR =complete remission, PD = progressive disease, SD = stable disease.

We examined if consecutive vaccination with an HLA-A2-restricted WT1-peptide would have an impact on the humoral immune response against the WT1 protein. The analyses of WT1-antibodies were performed in cooperation with Olga A. Elisseeva in Osaka, Japan.

Humoral responses against WT1 were analysed at baseline in 14 patients and during the course of vaccination in week 10 (n=8), in week 18 (n=7) and week 26 or 30 (n=6) by dot-blot analyses. At baseline IgM responses, defined as an IgM-titer ≥ 900 units were detected in 2 out of 14 patients available for WT1 antibody responses (Figure 16 A). An induction of anti-WT1-IgM antibodies was detected in 2 out of 7 patients at week 18 and in 6 out of 6 patients at weeks 26 or 30 during the time course, but never earlier. This effect correlated with improved clinical outcome according to progression-free survival (PFS) (Figure 16).

Spontaneous IgG responses at baseline were recorded in 3 out of 14 patients (Figure 16 B). In contrast to the IgM responses, no WT1-specific IgG antibody responses could be induced or enhanced by vaccination. The inability of induction of WT1-specific IgG antibodies might be related to the lack of specific T-helper cell stimulation. This hypothesis is underlined by the inability to detect functional WT1-specific CD4 T cells by intracellular cytokine staining (data not shown).

4.1.2 Vaccination of leukemia patients is able to induce both, KLH-specific IgM and IgG responses

As an antigen-independent CD4-helper cell stimulus *Keyhole Limpet Hemocyanin* was used as adjuvant in the vaccine.

Patient	IgM				IgG			
	pre	W10	W18	W26/30	pre	W10	W18	W26/30
13	0.45	0.66			0.54			
14	0.58	0.74			0.90	0.99		
15	0.64	0.44			1,63	0.98		
16	0.43		0.42		0.61		0.95	
17	0.52			0.67	0.59			1,01
18	0.48	0.46	0.49		0.37	0.93	0.82	
19	0.50	0.44			0.98	1,04		
20	0.58	3,11			0.73	0.97		
21	0.71		1,49		0.77		0.84	
22	0.52	0.71			0.75	0.68		
23	0.75	1,06		0.45	0.66	0.65		0.72
24	1,13	0.66	0.63	0.55	0.74	1,03	1,07	0.90
25	0.48	0.52	0.41	0.39	0.56	1,22	1,56	1,58
26	0.52		0.52	0.59	0.51		0.99	1,13

Table 17: KLH-specific antibody profiles in PB. Values ≥ 0.7 for IgM and values ≥ 0.8 for IgG were considered positive (mean of healthy donors + 2 fold SD)

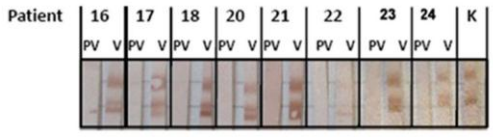
Because of the "non-self"-status and to assess if the vaccine-induced humoral response against an entire protein differs from the humoral responses against a small class-I-peptide epitope, the humoral anti-KLH response was analyzed. At baseline 3 out of 14 patients exhibited spontaneous KLH-specific IgM responses, defined as $E_{550} \geq 0.7$ (Table 17). Following vaccination enhanced KLH IgM antibody responses were detected in 4 out of 10 patients in week 10 and additionally in 1 patient in week 18.

Spontaneous IgG responses to KLH were measured in 3 out of 14 patients at baseline. In week 10, 4 out of 9 patients showed an induction of KLH-specific IgG-antibodies, whereas 4 out of 4 patients had enhanced KLH-specific IgG-antibodies in week 18 or 26.

4.1.3 Consecutive vaccination of leukemia patients leads to the induction of neutralizing GM-CSF-specific IgM and IgG antibodies.

In our setting, GM-CSF acts as a chemo-attractant for different kinds of immune cells, especially dendritic cells (see also Figure 4). To conclude the investigations on humoral immune responses we also performed experiments to determine if the administered hGM-CSF also leads to the induction of specific antibodies.

A



B

Patient	IgM			IgG		
	pre	W10	W26/30	pre	W10	W26/30
15	-	-	-	-	+	-
16	-	-	-	-	-	+
17	-	-	-	-	-	*
18	-	-	-	-	-	+[Π]
20	-	-	-	-	-	+
21	-	-	-	-	-	+
22	-	+	-	-	-	+
23	-	-	-	-	-	+
24	-	-	-	-	-	+
26	-	+	-	-	-	+

Figure 17: Specific anti-GM-CSF antibodies in sera of patients at different time points during vaccine course. a) Assorted Western-Blots, revealing the presence of GM-CSF-specific IgG-antibodies in sera of patients. PV = pre-vaccine; V = in vaccination; K = positive control. **b)** Positive sera (anti-IgA-IgG-IgM-antibody) were further classified into IgG and IgM isoformes. 10/26, week 10/26 after first vaccination;*positive for IgG-subtype at week 46; [Π]only positive for IgA-IgG-IgM-subtype

Western-Blot-analyses were performed in 14 patients at baseline as well as in week 10 and weeks 26 or 30. Serum of a rhesus macaque, previously described to contain anti-hGM-CSF antibodies [260] was used as positive control (Figure 17 A). At

baseline none of the patients' sera showed any Ig-reactivity against hGM-CSF. Anti-GM-CSF-IgM-antibodies were observed in 2 patients in week 10; IgG-responses could be detected in a total of 10 out of 14 patients (Figure 17 B).

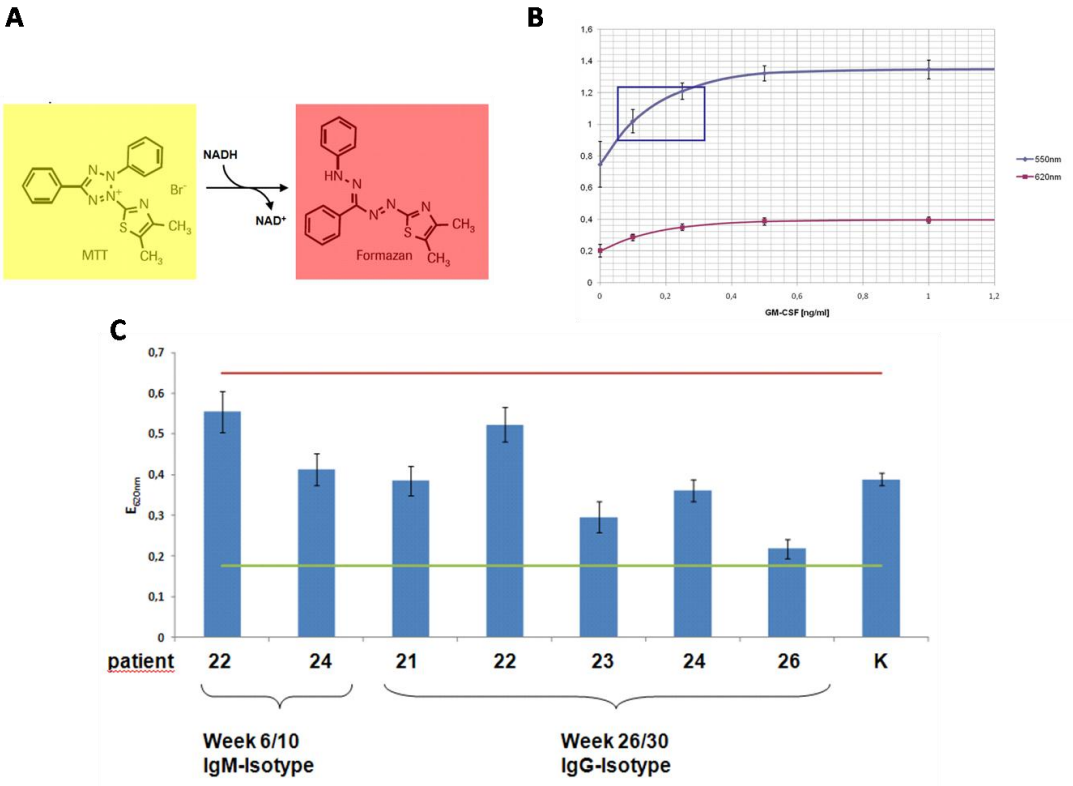


Figure 18: Detection of neutralizing anti-GM-CSF-antibodies a) Mechanism of the MTT-Assay. MTT is reduced to formazan by viable cells, leading to a color change, detectable via spectrometry. **b)** Preliminary experiment to determine an adequate GM-CSF concentration. The blue box indicates a GM-CSF range with maximal ΔE/ΔGM-CSF. **c)** Identification of neutralizing antibodies in sera of several patients. The red line indicates the uninfluenced growth of TF-1 cells with 2,5 ng/ml GM-CSF. The green line indicates the growth of TF-1 cells deprived of GM-CSF. Error-bars indicate the mean of a triplicate experiment.

Interestingly, following anti-tumor vaccination, three patients developed accompanying dry cough without radiologic abnormalities, which theoretically could be attributed to pulmonary alveolar proteinosis (PAP), a medical condition characterized by accumulation of surfactant in alveoli, which might be related to anti-GM-CSF autoantibodies.

To investigate the neutralizing potential of the GM-CSF-antibodies we performed a MTT-Assay (Roche)[263, 264], using TF-1 cells which proliferate dependently on GM-CSF, IL-3 or erythropoietin. MTT is internalized in viable cells and is reduced via $NADH^+$ to a stable salt, called Formazan. This chemical transformation is accompanied by a color switch (Figure 18a) which can be detected photospectrometrically. By incubating TF-1 cells under different GM-CSF

conditions (Figure 18b) we identified an adequate GM-CSF concentration of 2.5 ng/ml below which a minimal ΔGM-CSF would result in a maximal ΔE.
As displayed in Figure 18c, neutralizing activity of 30% of anti-GM-CSF sera could be demonstrated in patients 21, 23, 24 and 26 when using the monkey serum as reference value. Interestingly, in none of the patients in which dry cough occurred neutralizing anti-GM-CSF antibodies could be detected.

4.2 Phenotypical and functional analyses of cellular immunity in AML patients vaccinated with WT1-peptide plus GM-CSF and KLH

In the context of the phase I/II study with WT1-peptide-vaccination in AML patients we could previously demonstrate immunological and clinical responses but did not observe a correlation between T cell responses and outcome parameters in the initial analyses of WT1-specific tetramer staining and intracellular cytokine staining for TNF-α and IFN-γ [93]. Therefore more detailed immunological analyses were performed in a subgroup of patients. In detail, phenotypic charcterisation of CCR7 expression, induction of PD1 and CD137, and the cytokine profile by analyzing the capacity to produce IFN-γ, TNF-α and IL-2 and to mobilize CD107*a*, in response to the WT1 vaccine peptide were evaluated directly *ex vivo*.
In neither WT1 specific $CD3^+$ $CD8^+$ T cells nor in the whole $CD3^+$ T cell population a significant correlation could be found between cytokine or cytotoxic response or the activation marker CD137 and clinical outcome.

4.2.1 Differences in clinical outcome can be correlated to the T cell exhaustion marker PD-1

When evaluating the PD-1 data, we observed, that increased PD-1 values at baseline could be associated to influence PFS (p=0.05). Patients with PD-1 values exceeding 0.6% of CD3+CD8+ T cells (n=9; mean= 1.937%; median=0.88%; range 0.6% - 6.01%) at baseline exhibited a median PFS of 132 days (range= 49 -201 days) while patients with low PD-1 frequencies (n=7, mean= 0.25%; median= 0.16%; range 0.058% -0.2%) exhibited a significant longer PFS of 149 days (range= 57 -1682(+) days) (Figure 19 A).

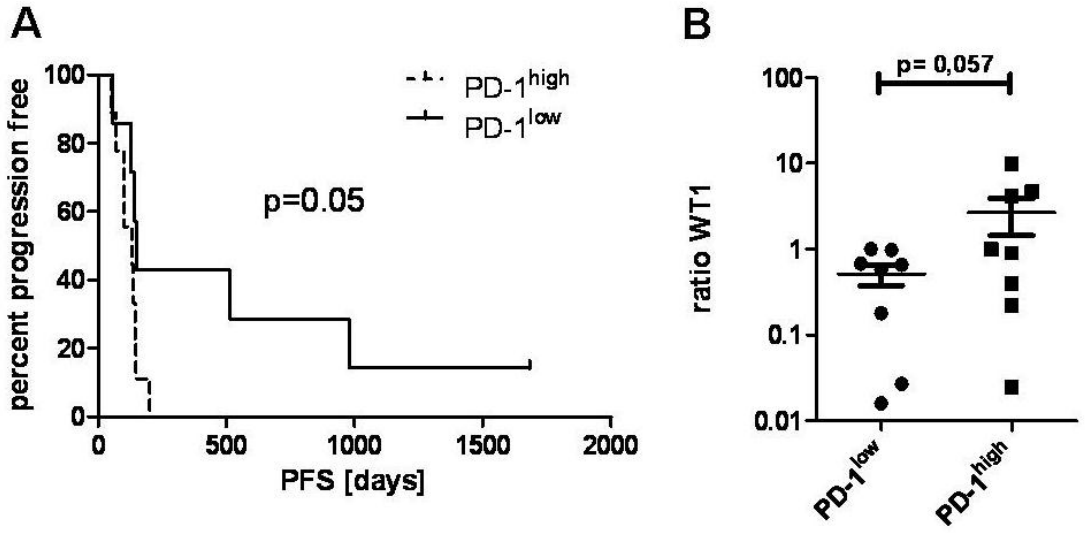


Figure 19: Correlation of PD-1-levels and progression-free survival (PFS). A) A shorter PFS can significantly be attributed (p=0.05) to the subgroup of patients with high PD-1 expression. **B)** Scatter-Plot of WT1-mRNA ratio distribution in both subgroups. High values of PD-1 expression are correlated to increased WT1-mRNA level.

Another trend, sustaining the reduced vaccine efficacy elicited by PD-1 can be seen when comparing the same groups in relevance to the differences in WT1-mRNA-levels at baseline and at week 10 of vaccination therapy as calculated on ($\text{ratio WT1} = \frac{WT1_{W10}}{WT1_{W0}}$). The patient subset exhibiting low quantities of PD-1 exhibited an average reduction of the WT1-mRNA level (range = 0.016 – 1; mean = 0.514; median = 0.62), while the patient subset with elevated PD-1 levels displayed an average increase in WT1-mRNA expression (range = 0.025 – 10; mean = 2.681; median = 0.95, p=0.05) (Figure 19 B).

4.2.2 Variations of CCR7$^+$-T cell frequencies are associated with clinical outcome

To examine in which T cell subset, if at all, potential functional defects would occur, the maturation and homing marker CCR7 was costained in our cytokine profile analyses (see 3.9.1). In 16 patients CCR7-expression on the whole T cell population was evaluated at baseline and in week 10. The analyses showed two behavioral subsets of our patients on terms of CCR7 alterations during treatment (Figure 20). In one group CCR7-expression levels increased during vaccination, while in the other group CCR7-expression levels decreased during treatment. Our data revealed that, an

increase of CCR7$^+$ CD8$^+$ T cells in patients is significantly assoiciated with favorable clinical course.

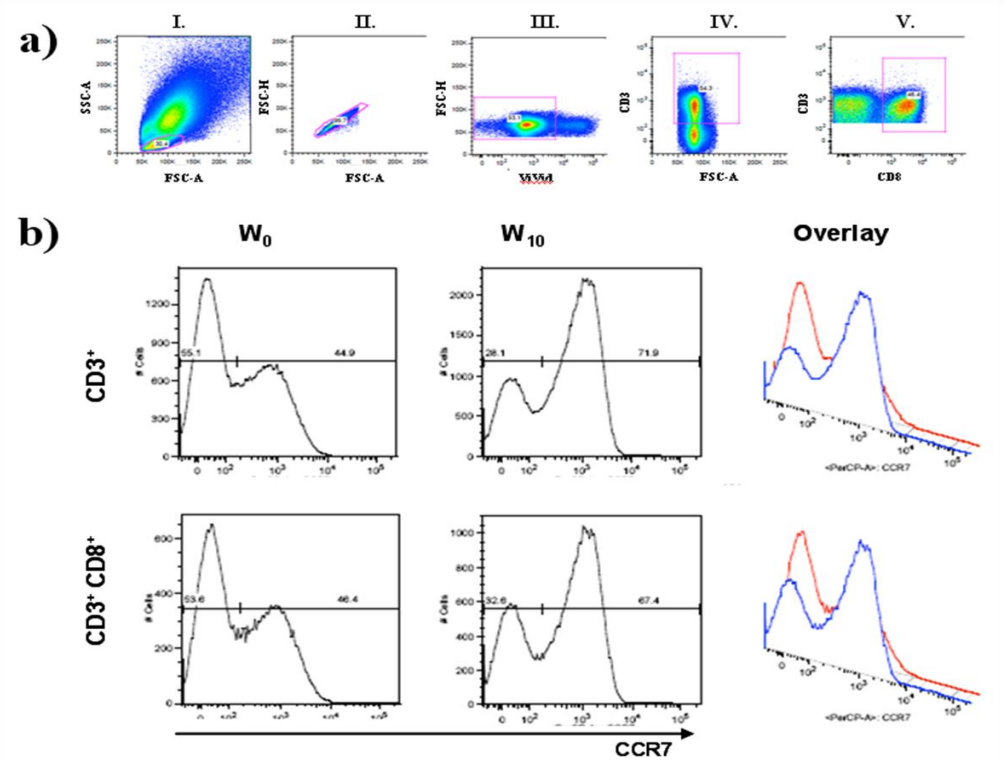


Figure 20: Determination of CCR7-frequencies. a) Gating strategy to determine CCR7-distribution: I.: lymphocyte gate; II.: singlet gate; III.: live/dead discrimination; IV: CD3$^+$ gate; V.: CD3$^+$CD8$^+$ gate. **b)** Exemplary CCR7 distribution of one patient in week 0 (W0) and in week 10 (W10), showing CCR7-frequency shifting in both, the CD3$^+$ CD8$^+$ - and the total CD3$^+$-population during time course.

Patients with an increase of CCR7$^+$ CD8$^+$ T cell frequencies (46.4% week 0 to 67.4% week 10) during vaccination had a significantly ($p<0.05$) longer PFS (median= 383 days, range 128-1682(+) days, n= 8) than patients with a decrease of CCR7$^+$ CD8$^+$ T cells (33.1% week 0 to 26.6% week 10) and a shorter PFS (median = 142 days, range 57-201 days, n=8) (Figure 21).

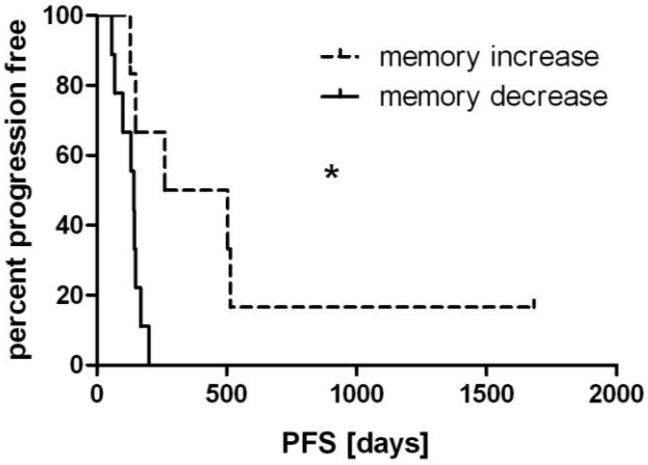


Figure 21: Kaplan-Maier-Plot showing differential progression-free survival of patient subsets. Patients with increase in memory cell frequencies exhibit significantly longer PFS than patients with reduced memory cell frequencies. * = $p \leq 0.05$

Evaluation of both subgroups on CCR7 surface expression did not reveal significantly different expression patterns of CCR7 at baseline ($p \geq 0.05$), but diverged significantly during vaccination ($p < 0.01$) in and between the subgroups (Figure 22 A). We also monitored the relation between the differential CCR7 surface expression during therapy and the matching WT1-mRNA ratio (Figure 22 B).

As seen in the corresponding graph, patients in whom CCR7-frequencies increase during vaccination exhibit a reduction of WT1-mRNA-levels in median as extrapolated by reduced WT1-expression levels in week 10 as compared to baseline. Nevertheless, this effect can not be scientifically determined, as statistics don't deliver confidence > 95% and therefore remains interpretative.

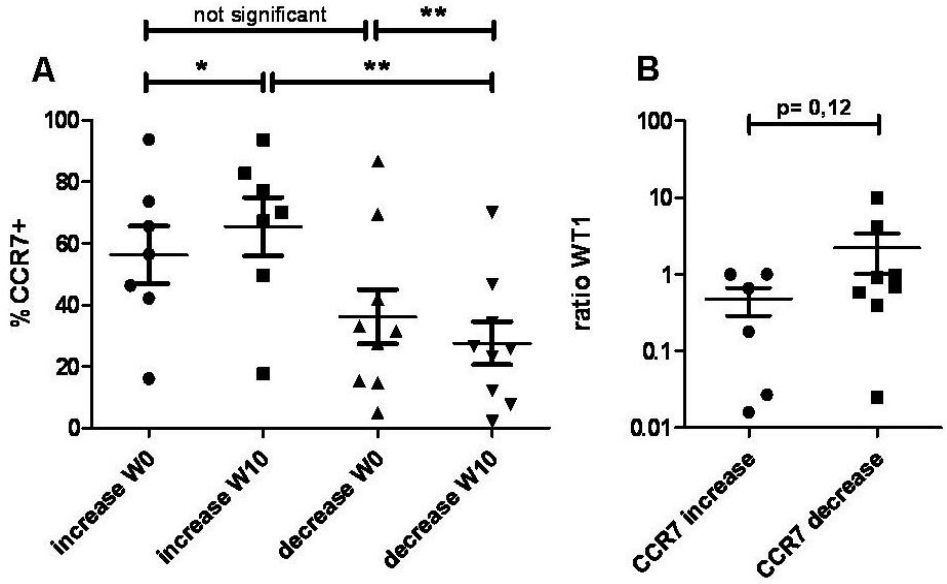


Figure 22: Correlation of CCR7-alteration and progression-free survival and WT1-mRNA expression. A) Differences in CCR7 surface expression display during vaccination. At baseline (week 0, W0) both subgroups don't exhibit significant differences in CCR7+ T cell frequencies, while the alterations in CCR7+ T cell frequencies in week 10 (W10) significantly differ in and between the subgroups. * = $p \leq 0.05$; ** = $p \leq 0.01$; ns = not significant. **B)** Scatter-Plot of WT1-mRNA ratio distribution in both subgroups. Increase of memory cells seems to be associated to reduction of WT1 mRNA during vaccination.

4.2.3 Clinical outcome is strongly associated to the interference of PD-1 and CCR7 with T cell function, but both factors act independently.

Since we could observe similar patterns concerning PD-1 and CCR7 expression in in relation to PFS, we plotted the patients with increased PD-1 levels as well as a decrease in CCR7 expression versus those in which one of these features was present and versus those who did not experience PD-1 induction and expressed CCR7 in

high amounts (Figure 23). We could observe a significant difference in PFS-values of the group with none of the detrimental markers (group A) to those which exhibited only one these markers (group B), as well as to those patients exhibiting both traits (group C). Overall PFS-values ranged from 49 to 1200 days (median = 143 days; mean = 312.5 days; n = 16). Group A exhibited a PFS range from 128 to 1200 days (median = 978 days; mean = 763.4 days; n=5) and was significantly elevated (p=** in both cases) compared to group B (PFS range from 49 to 149 days; median = 143 days; mean = 126.8 days; n = 6) and group C (PFS range from 57 to 201 days; median = 101 days; mean = 112 days; n = 5).

Therefore, in terms of PFS, it seems that the acquisition of one detrimental trait is sufficient to impair functional anti-tumor immunity and that T cell exhaustion and impaired homing capacity act independently.

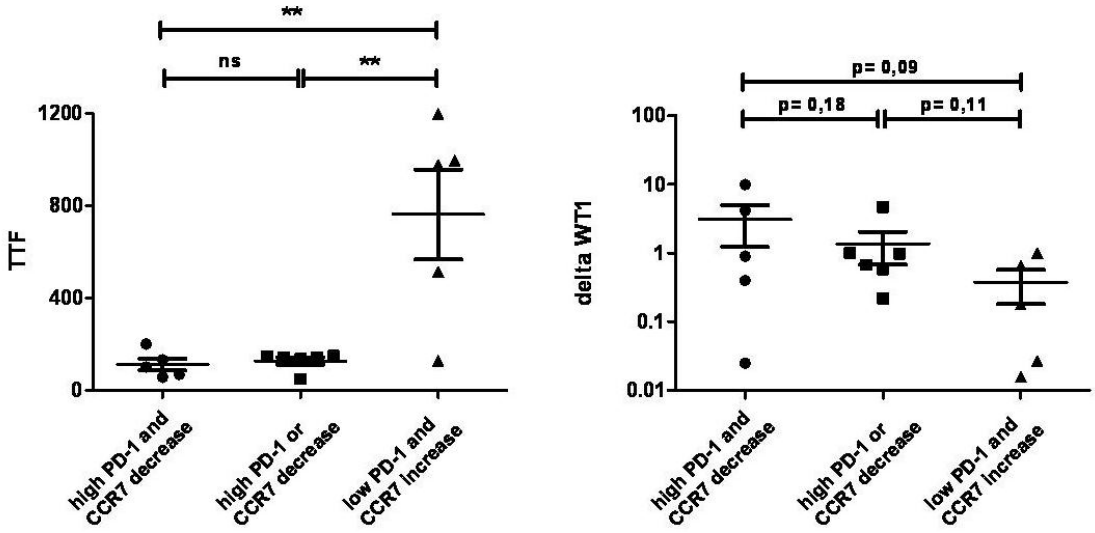


Figure 23: Effects of simultaneous acquisition of elevated PD-1 and decrease of CCR7-expression on PFS and WT1-mRNA expression. Left: simultaneous acquisition of high PD-1 levels as well as a decrease in CCR7-frequencies has no significant impact on PFS compared to the acquisition of a single characteristic. **Right:** in contrast to PFS, the declination of WT1-mRNA values seems to follow a sequential trend in patients, exhibiting both, only one or none of these attributes.* = $p \leq 0.05$; ** = $p \leq 0.01$; ns = not significant.

We also compared the same groups in order to determine potential associations of PD-1 and/or CCR7 to changes of WT1-mRNA during vaccination therapy (Figure 23). Though not significant, we observed buoyancy to WT1 increase with accumulation of these receptors. Group A exhibited a lower WT1-mRNA ratio range from 0.016 to 1 (median = 0.18; mean = 0.376; n = 5) compared to group B (range from 0.22 to 4.7; median = 0.83; mean = 1.36 days; n = 6; p = 0.11) or group C (range from 0.025 to 10; median = 0.9; mean = 3.1; n = 5; p = 0.09).

4.2.4 Differentiation states of WT1-specific T cells at baseline and during therapy are not mirrored in clinical outcome.

To further investigate if the differentiation status of WT1-specific T cells would be associated with vaccine efficacy, detailed phenotypic analyses were performed in 12 patients (Figure 24). Presence of a WT1-specific T cell at baseline were considered positive when they exceeded 0.3% of total CD3+CD8+ T cells, representing the mean value +2 SD of a control group; *c.f.* Keilholz *et al.* [93]. At baseline, all patients, except patient 6 (1.15 %), did not exhibit WT1-specific tetramer + T cells (mean = 0.2 %; range = 0.05% - 1.15%; Figure 24, column graphs). After 6 vaccinations, 8 out 12 patients exhibited an at least two-fold increase of initial WT1-specific tetramer + T cells (patients 1, 2, 10, 11, 18, 22, 25 and 26; mean = 0.51%; range = 0.14% - 1.09%), including 5 patients with significantly elevated levels when compared to the threshold value (patients 1, 2, 11, 22, and 25; mean = 0.66%; range = 0.041% - 1.09%).

In parallel to the flow-cytometric assessment of WT1-specific tetramer responses, the CCR7 and CD45 expression on the WT 1-tetramer-positive T cells was evaluated in the same experiment to allow discrimination of 4 main T cell subpopulations, naïve, central memory, effector memory and effector T cells (Figure 24, pie charts).
Phenotypic distribution of the different T cell subsets proved to be heterogeneous among the individual patients. At baseline, mean values of total $CD3^+CD8^+$ WT1-specific T cells appointed 24.7% to the $T_{naïve}$ subset (range = 2% – 46%), 11.7% to the T_{cm} subset (range = 0% – 41%), 12.1% to the T_{em} subset (range = 0% – 33%) and 51.3% to the T_{eff} subset (range = 5% – 86%), respectively. After 6 vaccinations, the total $CD3^+CD8^+$ WT1-specific T cells comprised mean values of 21.25% of the $T_{naïve}$ subset (range = 3% – 54%), 9.95% of the T_{cm} subset (range = 0% – 54%), 18.2% of the T_{em} subset (range = 1% – 84%) and 50.55% of the T_{eff} subset (range = 12% – 93%), respectively. However, no correlation could be found between variances in the phenotypic composition and clinical outcome in terms of PFS (data not shown). Interestingly, patient 6, with initially elevated frequencies of WT1-specific T cells, exhibited an almost constant level of WT1-specific T cells after 6 vaccinations accompagnied by an equally stable phenotypic constellation.

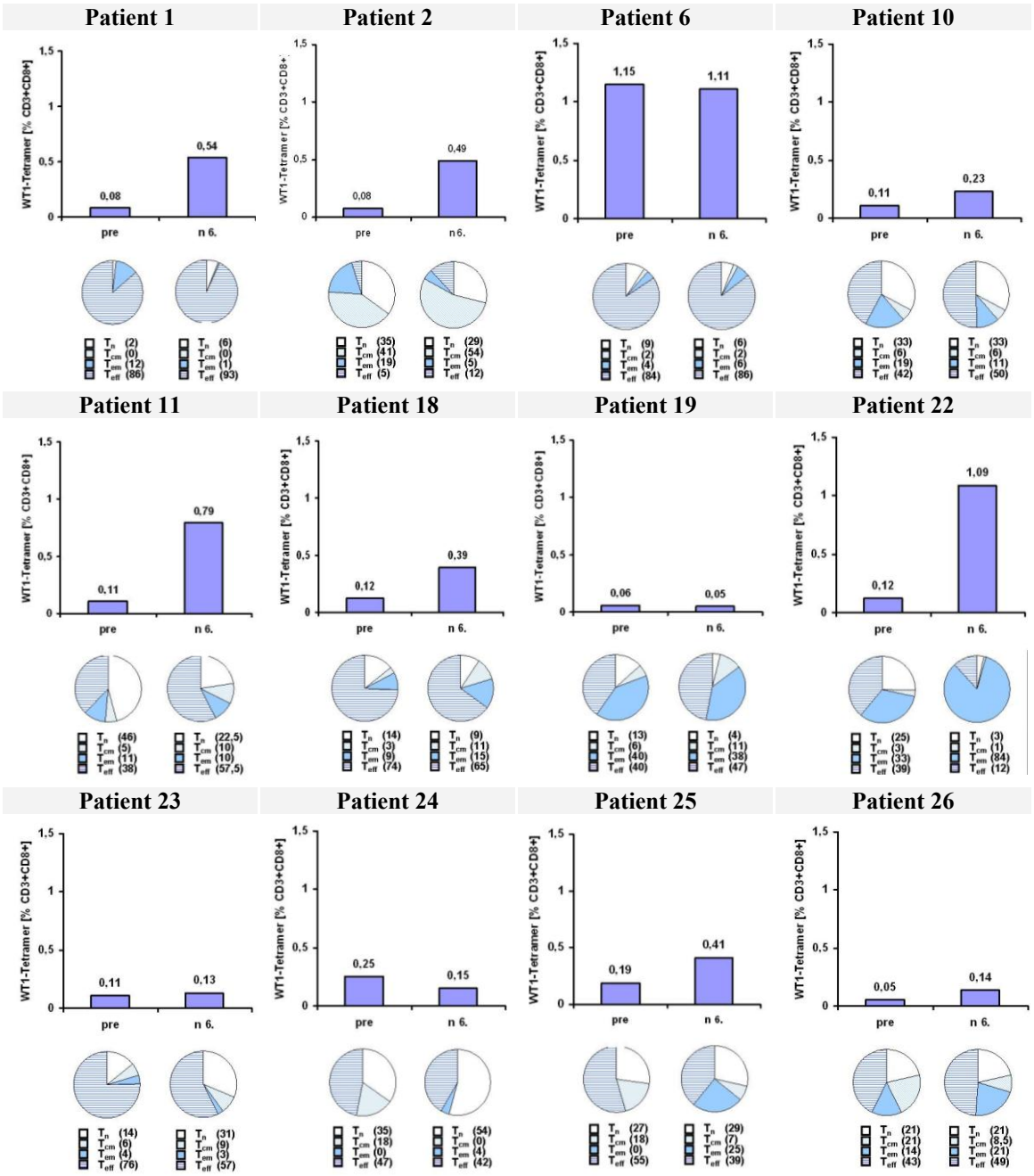


Figure 24: Presence and phenotypic composition of WT1-specific T cells in PB of selected patients. WT1 specific T cells were identified at baseline and after the 6th vaccination by WT1 specific tetramer staining of the CD3+CD8+ lymphocyte fraction (column graphs) for each patient. In parallel, the phenotypic composition of this specifc subset was assessed by CD45RA/CCR7-staining, identifying naïve (T_n), central memory (T_{cm}), effector memory (T_{em}) and effector (T_{eff}) T cells (pie charts).

Similar heterogenic variances were observed in the total lymphocyte fraction. The phenotypic distribution of the different T cell subsets at baseline showed mean values of total lymphocytes comprising 34.93% of the $T_{naïve}$ subset (range = 1.5% – 57%), 16.75% of the T_{cm} subset (range = 0.5% – 54%), 10.56% of the T_{em} subset (range = 2.5% – 15%) and 37.69% of the T_{eff} subset (range = 1.5% – 88%), respectively. After 6 vaccinations, the total lymphocytes accounted for mean values

of 30.19% of the $T_{naïve}$ subset (range = 5% – 70%), 13.25% of the T_{cm} subset (range = 0.5% – 28%), 11.62% of the T_{em} subset (range = 2% – 20%) and 44.81% of the T_{eff} subset (range = 12% – 86%), respectively (individual data not shown, but summarized in Figure 25 B).

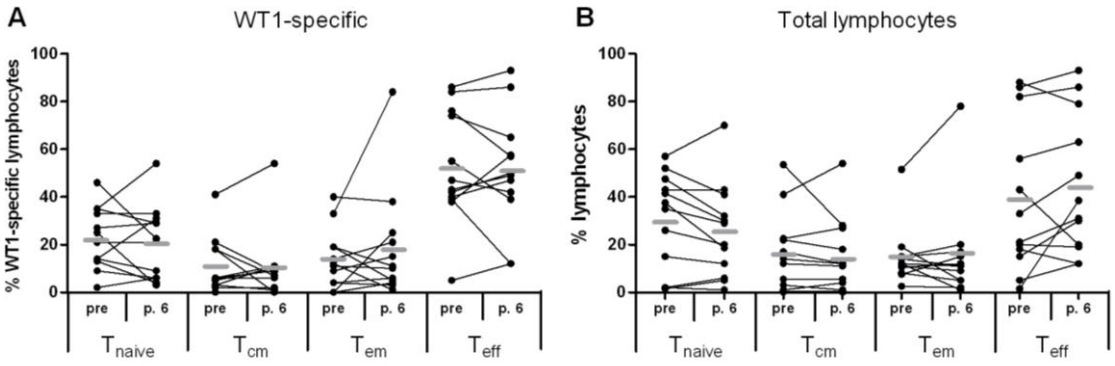


Figure 25: Proportions of different T cell subsets among WT1-specific T cells and total lymphocytes at baseline and after the 6th vaccination. A Percentages of the T cell subsets in the WT1-specific $CD3^+CD8^+$ T cell fraction acoording to CCR7/CD45RA-staining. **B** Percentages of the T cell subsets in the total lymphocyte fraction acoording to CCR7/CD45RA-staining. Mean values are indicated as grey bars.

Despite no general significant alteration in the proportions of the T cell subsets could be observed among the patients, significant intra-individual shifts occur in both, the WT1-specific $CD3^+CD8^+$- as well as in the total lymphocyte population (Figure 25). Nevertheless, the initial heterogeneity of the individual patients concerning their phenotypic composition does not allow any assumption about vaccine associated effects.

Due to their clinical performance (PFS ≥ 514 days; *c.f.* Table 6), additional long-term analyses could be performed in 4 of the 12 selected patients (patients 1, 2, 22 and 24, respectively).

Despite the initial increase in WT1-specific T-cells, patient 1 (PFS = 514 days) exhibited an oscillating pattern concerning the frequencies of WT1-specific T-cells. A boosting of WT1-specific T-cells after the 6th vaccination was followed by a consecutive reduction of WT1-specific T-cells at the next reading ? point (Figure 26). Interstingly this effect was accompagnied by a pattern in the phenotypic composition. Apparently, with already high levels of T_{EFF} cells at baseline, the initial boost of WT1-specific T-cells leads to the predominace of T_{EFF} cells and an almost complete depletion of WT1-specific- T_{EM} cells in this patient after 6 vaccinations. As T_{EFF} cells exhibit a limited lifespan, the low frequencies of WT1-specific T-cells after 10 vaccinations may be explained by the declination of the primary vaccine-

induced immune response. A similar picture could be observed concerning the timepoints after 15 and 20 vaccinations, respectively. After the 15th vaccination, the WT1-specific T-cells re-increased to a value comparable to the initial boost after 6 vaccinations, but declined again at the reading point after the 20th administration. Simultaneously, the phenotypic composition equally mirrored the composition observed at the initial boost with a predominace of T_{EFF}-cells and a depletion of T_{EM}-cells.

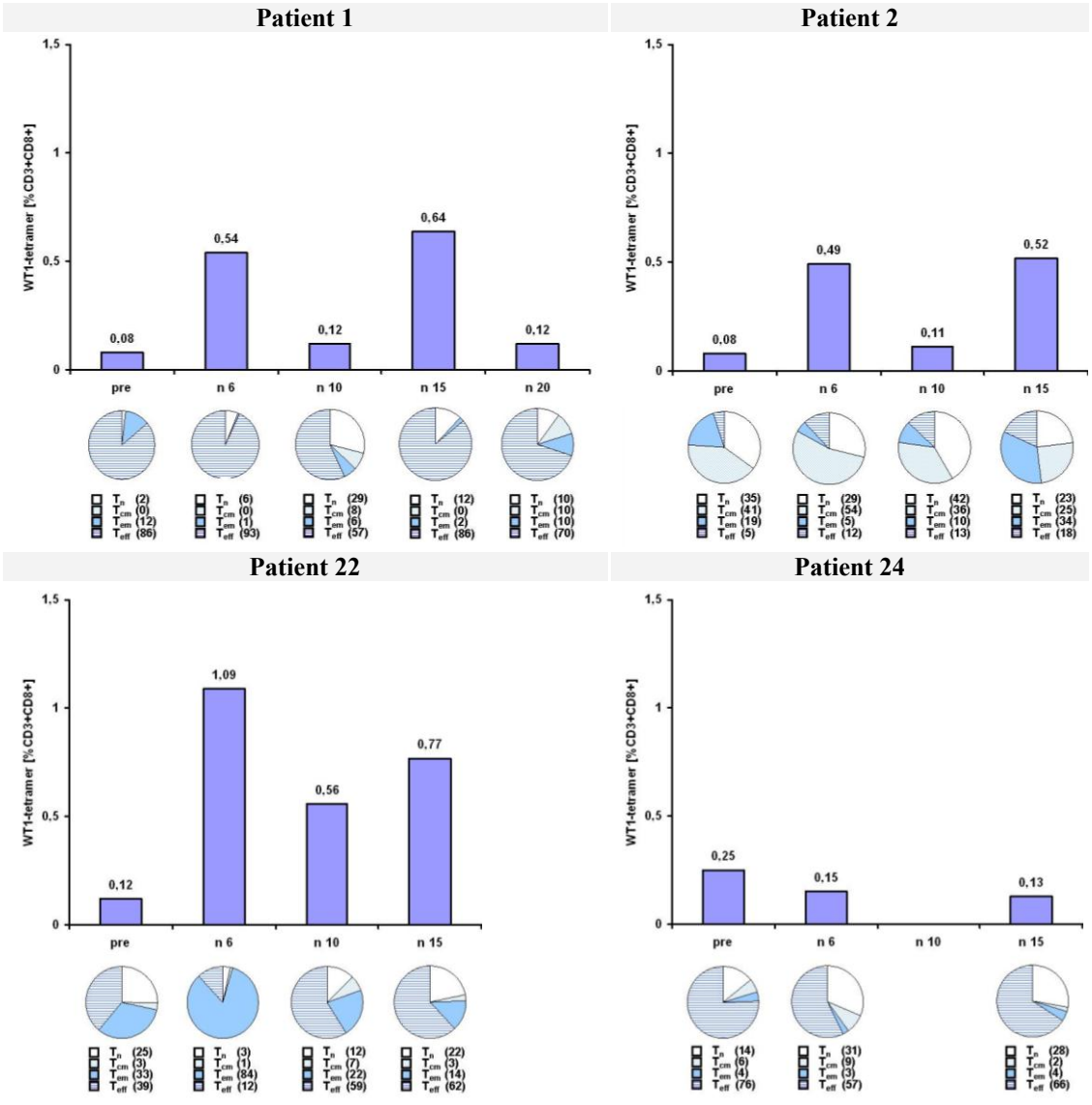


Figure 26: Presence and phenotypic composition of WT1-specific T cells in PB of long-performing patients. Complementing the initial analyses, WT1 specific T cells were additionally identified after the 10th, 15th and 20thvaccination, if material was available (column graphs). In parallel, the phenotypic composition of these subsets was assessed by CD45RA/CCR7-staining as performed before (pie charts).

While patient 2 (PFS = 978 days) exhibited similar features concerning the oscillation of WT1-specific T cells during vaccination, no associated pattern could be found when exmaning the phenotypic composition of the WT1-specific T cells.

As already presented in Figure 24, Patient 22 (PFS > 1682 days) exhibited initially low WT1-tetramer-frequencies. After the 6th vaccination WT1-specific T-cells significantly increased and constituted 1.09% of total $CD3^+CD8^+$- T cells. Throughout the treatment phase WT1-frequencies declined to 0.54% after the 10th vaccination but recovered to 0.77% after the 15th vaccination (Figure 26) and remained stable since (data not shown). This development was accompied by a high presence WT1-specific effector-memory T cells after the 6th administration, probably due to an occurring vaccine-primed anti-tumor reaction. As the amount of WT1-specific T cells levels off at a stable frequency, the phenotypic composition also seems to attain a new equilibrium with an increased fraction of effector T cells, indicating an ongoing active immune response against leukemic blasts. Nevertheless, the initially elevated T_{EM} equally remains stable, indicating the establishment of an immunologic memory and probably serving as a source for the permanent reservoir of tumor-directed effector T-cells.

Despite the unexpected long clinical and effective molecular response of patient 24, the immunologic response remained weak in relation to the beneficial clinical course in terms of PFS (> 1668 days). From vaccination initiation the patient exhibited only weak frequencies of WT1-specific T cells.

4.3 Influence of immune regulatory cells

4.3.1 Regulatory T cell frequencies in AML patients are significantly elevated, but time-dependent variations in T_{reg}-frequencies can not be correlated to prognosis

As mentioned above, several groups have described an influence of regulatory T cells on proper T cell functions in the context of anti-tumor immunity. For this reason we screened our patients for the presence of peripheral T_{regs} (Figure 27).

Regulatory T cells in peripheral blood were assessed by flow cytometry and defined as $CD25^+CD127^{low}$ cells (Figure 27 A). Although this definition predominantly

applies to $CD4^+$ T cells, we expanded this definition to equally screen for potential regulators in the $CD8^+$ and the total $CD3^+$ fractions.

In general, T_{reg}-frequencies remain stable over time in the vaccinated AML patients. There are some distinct outliers, in which Treg frequencies vary significantly, but the low number of these special cases does not allow any assumptions of specific vaccine-associated effects. AML-patients exhibit significantly elevated frequencies compared to those of healthy volunteers (Figure 27 B). $CD25^+CD127^{low}$ – frequencies in the $CD4^+$ fraction of AML patients oscillate around 6% of the total $CD3^+$-fraction (W_0: mean = 6.27%; median = 5.33%; range: 0.33% - 14.67%; W_{10} : mean = 6.01%; median = 5.47%; range: 0.39% - 15.74%; n= 16), while healthy individuals exhibit lower levels of T_{reg} (mean = 2.14%; median = 2.62%; n = 5) in a condensed range (0.72% - 2.98%).

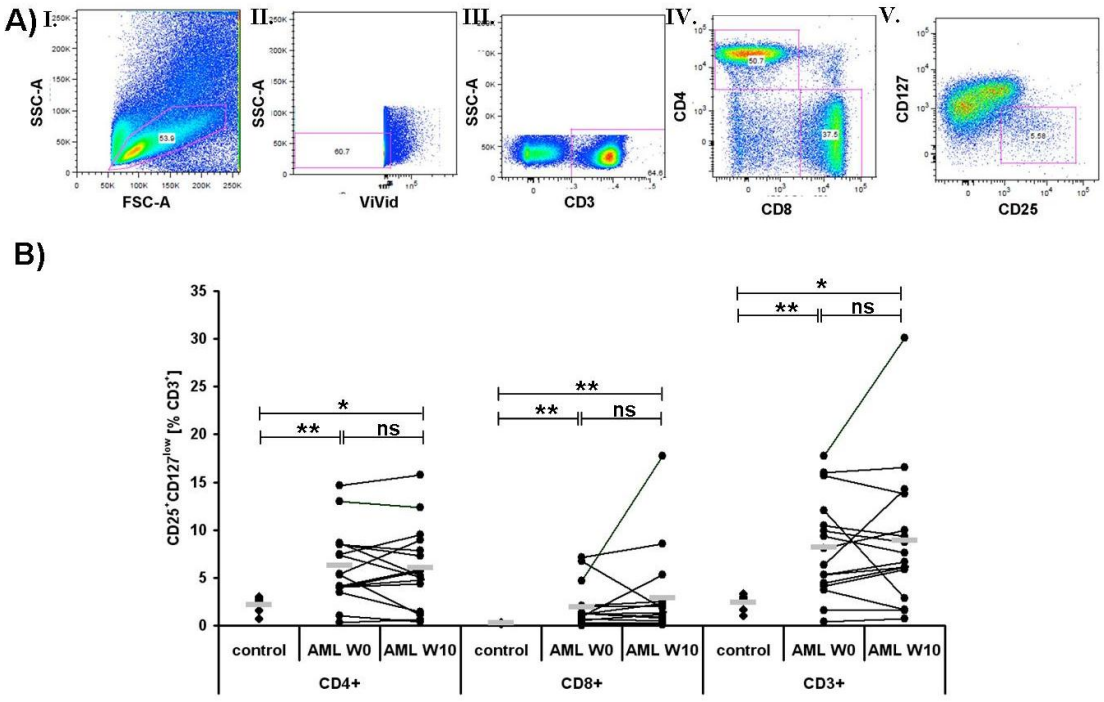


Figure 27: Assessment of T_{reg} –frequencies. A) Gating strategy to identify regulatory T cells: I.: lymphocyte gate; II.: live/dead discrimination; III: $CD3^+$ gate; IV.: $CD4^+$ and $CD8^+$ gates; V.: $CD25^+CD127^{low}$ -subset **b)** Distribution of regulatory T cells in peripheral blood of controls and AML patients during vaccination. Scatter-plots show $CD25^+CD127^{low}$ frequencies in terms of [$\%CD3^+$]. Plots were generated for $CD4^+$ and $CD8^+$ fractions of $CD3^+$ lymphocytes, as well for the cumulative values (designated $CD3^+$) of both subsets. Mean values are displayed as grey bars. * = $p \leq 0.05$; ** = $p \leq 0.01$; ns = not significant.

The same effect holds true for $CD8^+CD25^+CD127^{low}$ T_{regs}: significant differences of AML patients in comparison to healthy controls, but also no alterations during vaccination course. However, it is noteworthy, that we are able to describe elevated levels of $CD8^+CD25^+CD127^{low}$ cells in our AML patients. To our knowledge, this

phenotype has not been described previously in AML. Healthy controls only exhibit very low numbers of the specified phenotype (mean = 0.20%; median = 0.16%; range: 0.12% - 0.33%). In contrast, AML patients show distinct populations of $CD8^+CD25^+CD127^{low}$ T_{regs} (W_0: mean = 1.9%; median = 1.19%; range: 0.019% - 7.12%; W_{10}: mean = 2.85%; median = 1.28%; range: 0.04% - 17.76%).

4.3.2 Myeloid derived suppressor cells are elevated in AML patients vaccinated with WT1, but have no impact on clinical outcome

In the context of cellular immune suppressive factors we also screened our patients for the presence of peripheral myeloid derived suppressor cells at baseline and in week 10. MDSC were characterized as $CD11^+CD14^+HLA\text{-}DR^{-/low}$ cells that did not express lymphoid markers (CD3, CD19 and CD56).

We could observe a significantly elevated amount of MDSC in our patients at baseline (mean = 1.78%, range = 0.005% - 10.42%; median = 0.66% n=16) compared to healthy donors (mean = 0.36%, range = 0.027% - 1.68%; median = 0.28% n=19) with a confidence interval > than 95% (Figure 28). During vaccination, a trend aroused in regards of a drop of the mean frequencies of MDSC in the AML patients (W10: mean = 0.91%, range = 0.012% - 5.36%; median = 0.49%; p=0.08; n=16), but could not be proven statistically (p = 0.08). Despite the potential reduction of MDSC in peripheral blood of AML-patients, the median level remained statistically elevated compared to the control group.

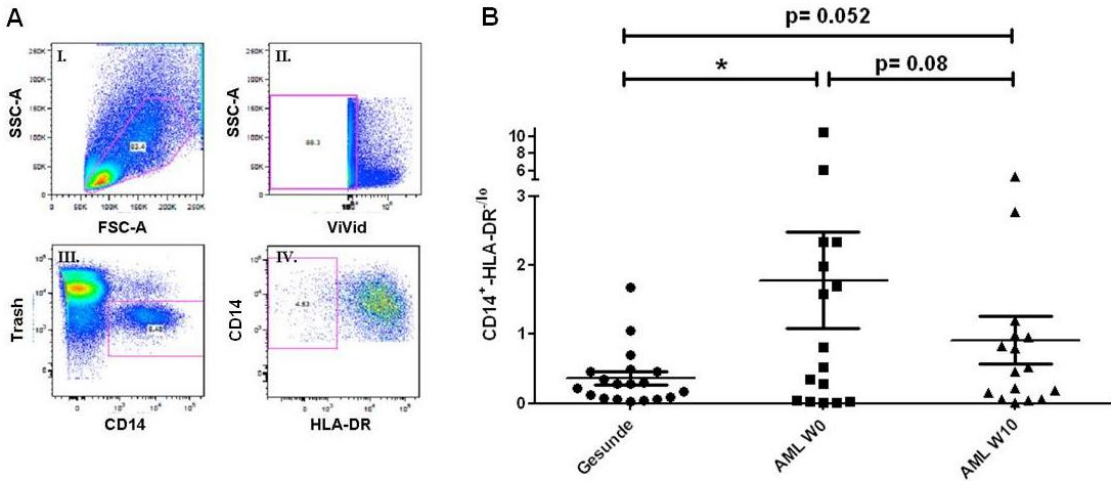


Figure 28: Evaluation of MDSC –frequencies in PB. A) Gating strategy to identify MDSC: I.: lymphocyte gate; II.: live/dead discrimination; III.: $Trash^-$ $CD14^+$ gate; IV.: $CD11^+$ $HLA\text{-}DR^{-/low}$ gate; **B)** Distribution of myeloid derived suppressor cells in peripheral blood of AML patients during vaccination and controls. * = $p \leq 0.05$.

Additionally, MDSC frequences were analysed at timepoint of relapse in 15 patients. In 10 patients MDSC frequencies were analysed in BM and in 5 patients PB-samples were available for MDSC-frequency assessment. In comparison to the same compartment at week 0, baseline values were comparable to those of the preliminary, pure PB-screening (mean = 1.28%, range = 0.008% - 7.6%; median = 0.41% n=15). MDSC-frequencies were slightly elevated at time of relapse but did not significantly differ compared to baseline (mean = 1.464%, range = 0.027% - 4.74%; median = 0.93%; p=0.08; n=15) due to the two outliers at baseline (Figure 29).

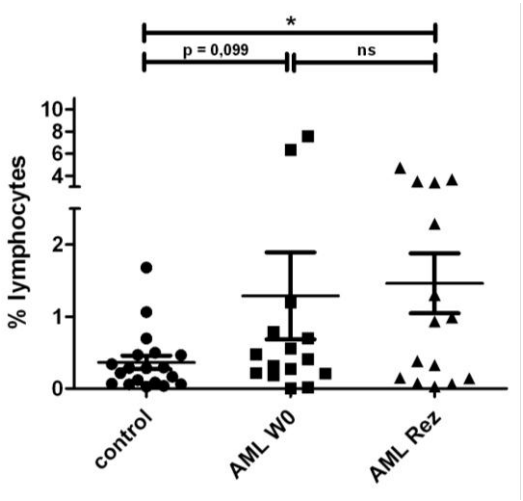


Figure 29: Evaluation of MDSC-frequencies at relapse. Despite elevated levels of MDSCs at relapse, these frequencies do not significantly differ from those at baseline. * = $p \leq 0.05$; ns = not significant.

4.3.2.1 T_{regs} and MDSC are not linked in a common regulatory network in patients vaccinated with WT1

As it has been described that MDSCs and T_{regs} may be interacting through a common regulatory network [231, 265], we also examined if the individual frequencies of both cell subsets would be related during vaccination. As seen in (Figure 30), no significant association could be found between the variances in T_{reg}- and MDSC-frequencies.

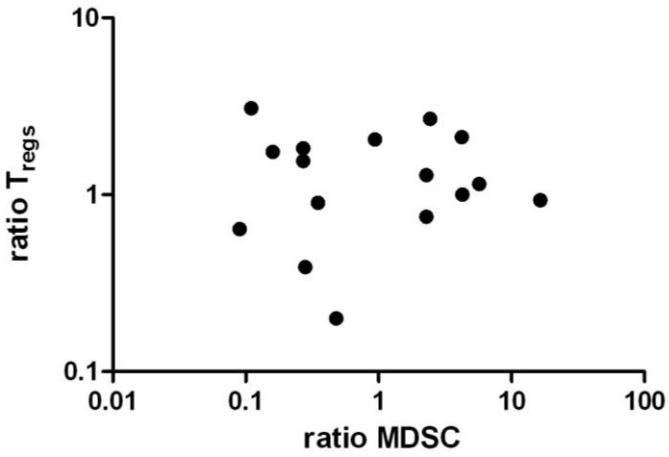


Figure 30: Correlation of MDSC- and T_{reg}- frequencies. Variances in T_{reg}-frequencies during therapy were plotted against the corresponding variances in MDSC-frequencies of the individual patients. In our specific context, MDSCs and Tregs do not seem to interact in a common regulatory network.

4.4 Expression of immune modulators on leukemic blasts

Despite initial benefit in response to the vaccine, several patients relapsed (median PFS= 139 days). To investigate if the leukemic blasts contribute to immune regulatory effects, the expression of potential leukemic immune-modulators on the blasts, including PD-L1, INDO, PI-9, HMOX, IL-10, CXCR4 and TGF-β, was examined by qRT-PCR at baseline and at time of relapse. A total of 15 patients were screened for altered expression of these factors, either in PB (n=5) or BM (n=10) for both timepoints. Samples of healthy subjects (n=5), of patients with different solid tumor entities (n=14) and of untreated AML patients with high leukemic burden (n=5) served as control-groups.

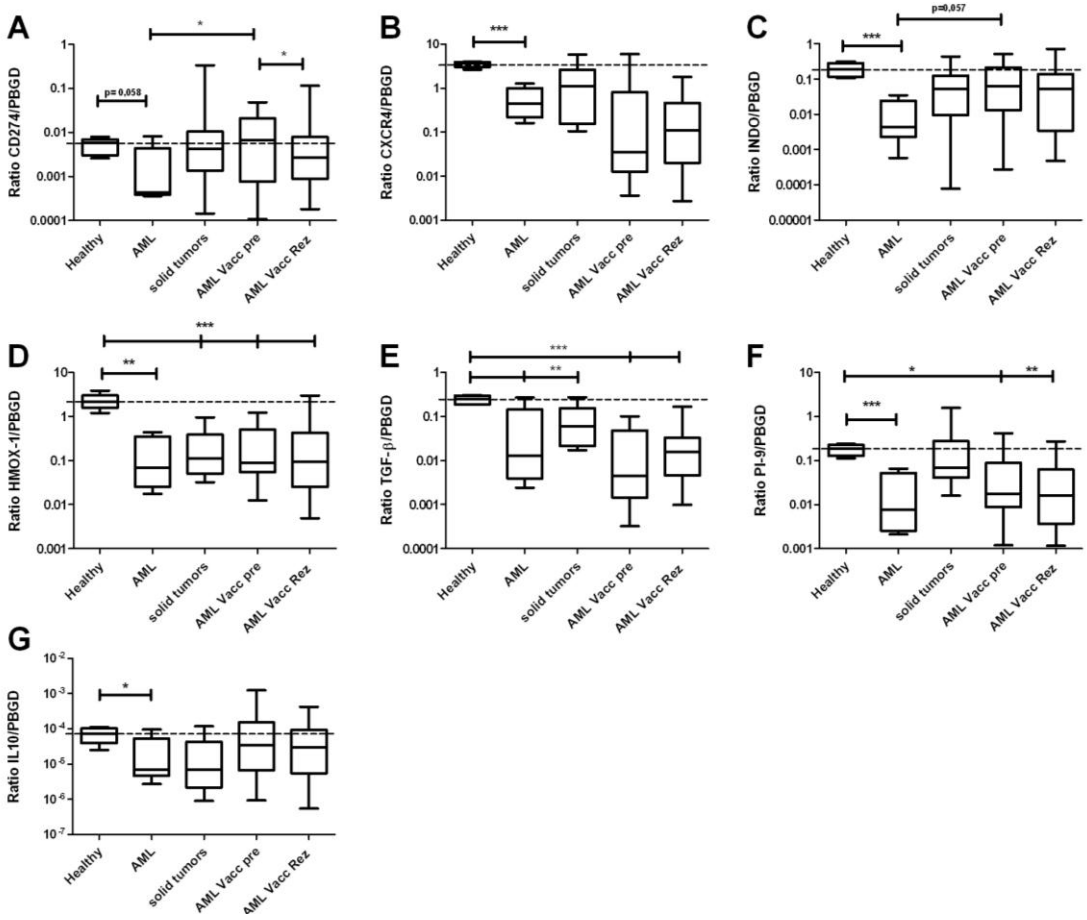


Figure 31: Whisker Plots showing alterations in the expression of different immune modulators in samples of vaccinated AML-patients at two distinct time points compared to control groups. Vaccinated AML patients were compared to healthy controls, untreated AML-patients and to a compound of different solid tumors (Table 14) for elevated expression of CD274, TGF- β, HMOX CXCR4, IL-10, PI-9 and INDO (A through G). The dotted line represents the mean value of the healthy control group. Interestingly, only the immune modulator CD274/PD-L1 is significantly altered when comparing expression in week 10 to that at baseline (A). No further significant alteration, at least between the two vaccine time points, can be observed (B-G). * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; ns = not significant.

For all immune modulators a significant higher expression in relation to the house-keeping gene PBGD was observed in healthy subjects when compared to the untreated AML patients (range: p= *** to p= 0.058, Figure 31 A through G). Significant differences in expression between untreated and vaccinated patients prior to vaccination were only detected for of PD-L1 and INDO (p= * and p= 0.057, respectively; Figure 31 A and C).

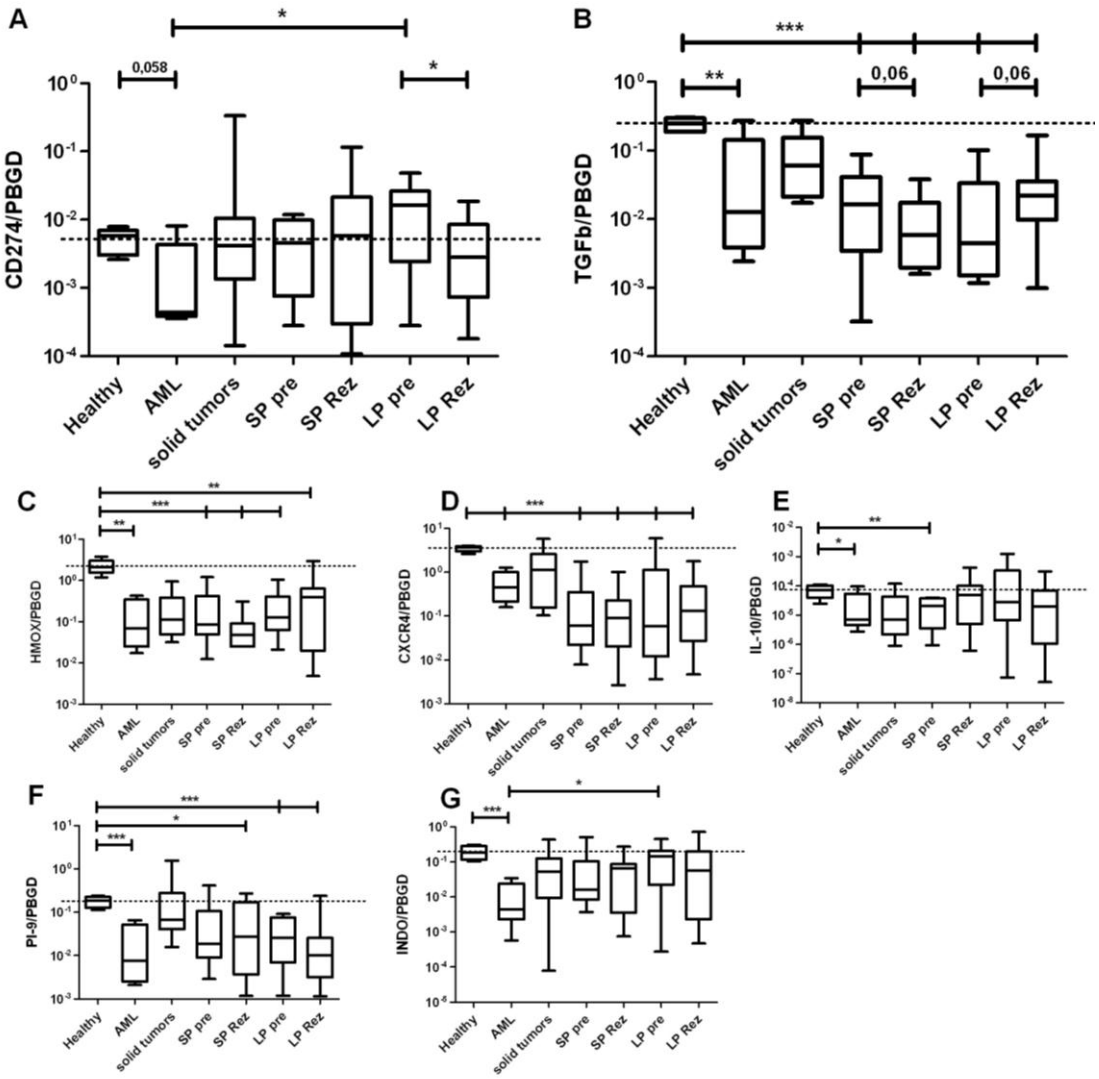


Figure 32: Whisker Plots showing alterations in the expression of different immune modulators in samples of vaccinated AML-patients at two distinct time points and PFS-subsets (LP, SP) compared to control groups. Vaccinated AML patients were compared to healthy controls, unvaccinated AML-patients and to a compound of different tumor entities for elevated expression of CD274, TGF- β, HMOX CXCR4, IL-10, PI-9 and INDO (A through G). The dotted line represents the median value of the healthy control group. A) CD274 expression varies significantly in a group of patients that exhibits a longer PFS (L_{PFS}, > 139 days, p= *). Interestingly no oppositely regulated process appears in the S_{PFS} subgroup. B) Differential expression of TGF- β eypression can be observed between both subgroups. (C-G) As in the previous figure, no further significant alteration, at least between the two vaccine time-points, can be observed. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; ns = not significant.

Concerning the intra-regimental relation, only PD-L1/CD274 (Figure 31 A) showed a significantly altered expression (p= *) at baseline (median = 0.0067; mean = 0.013) and at the time point of relapse (median = 0.0027; mean = 0.012).
This is of special interest, since in section 4.2.1 we describe a significant correlation of the PD-L1-receptor PD-1 with progression-free survival.
As additional analyses we subsequently divided the 15 patients into two subsets, long (L_{PFS}) and short progression-free survival (S_{PFS}), according to the median PFS (>139 days, n=9; ≤139 days, n= 6; respectively) and revaluated.
As a result, a significant decrease in PD-L1/CD274 expression during vaccination could be correlated to a higher PFS (p= *; Figure 32 A). Prior to vaccination the long performing subset of patients exhibited an increased level of PD-L1 (median = 0.016; mean = 0.017) in relation to the healthy control subset (median = 0.006; mean = 0.005). At the time-point of relapse, the long performing subset exhibited values of PD-L1 expression close to that of healthy controls (median = 0.003; mean = 0.005). In contrast, the S_{PFS} subset showed no alterations. Even though the means of both time-point differ (baseline = 0.005; relapse = 0.021), median values stayed similar to that of the healthy control subset (baseline = 0.005; relapse = 0.006).
A second interesting aspect is the opposite regulation of TGF-β expression in the short- and long PFS subsets (Figure 32 B). While the S_{PFS} subset exhibited a decrease in TGF- β during vaccine administration (baseline: mean = 0.025; median = 0.017; relapse: mean = 0.01; median = 0.005; p = 0.067), the L_{PFS} subset presented an increase in TGF- β expression (baseline: mean = 0.02; median = 0.0044; relapse: mean = 0.03; median = 0.02; p = 0.059).

4.5 Summary of individual immune modulative mechanisms in the patient collective

Our data suggests that, at least in our setting, vaccine efficacy is not influenzed by a single specific immune modulatory mechanism, since we can not observe any significant correlation of the selected immune modulators to clinical outcome. Nevertheless, while theses modulators do not exert a general effect, their presence and induction in individual cases surely may influence the individual outcome of the single patient. For this reason we summarized the individual parameters to gain insight in the distribution of these parameters among our patients (Table 18).

	WT1-Panel				Immunmodulatory								
	FACS				PCR							FACS	
Patient	ΔCCR7	PD-1	ΔT_{reg}	ΔMDSC	ΔCXCR4	ΔTGF-β	ΔIL-10	ΔINDO	ΔCD274	ΔHMOX	ΔPI-9	ΔMDSC	PFS
1	1.09	Low (0.06%)	0.64	0.09	0.97	1.61	0.41	0.23	0.17	0.38	0.48	0.68	514
2	0.99	Low(0.2%)	1.29	2.29	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	978
3	n.a	High (1.15%)	2.69	2.44	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	136
5	n.d.	n.d.	n.d.	n.d.	1.46	3.22	39.5	11.54	19.90	10.14	2.64	17.28	37
6	0.81	High (5.06%)	1.55	0.27	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	201
7	1.45	Low (0.42%)	1.75	0.16	0.04	0.11	6.22	16.78	0.49	0.11	2.47	4.68	128
8	n.a.	i.h. (2.09%)	1.15	5.70	n.a.	n.a.	1.26	0.13	n.a.	3.58	0.40	8.44	49
9	n.d.	n.d.	n.d.	n.d.	23.90	16.71	0.12	0.02	0.03	2.37	0.55	0.12	310
10	0.81	n.d.	n.d.	n.d.	1.01	0.84	0.70	0.53	1.06	0.26	1.10	41.77	262
11	0.78	High (3.83%)	2.05	0.94	1.89	1.98	5.32	1.43	1.92	2.03	1.65	0.29	101
12	0.67	High (1%)	0.75	2.29	0.94	3.99	0.11	0.30	0.23	1.41	0.04	0.49	144
15	0.80	Induced (3x)	2.12	4.22	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	57
17	n.d.	n.d.	n.d.	n.d.	35.57	3.99	28.18	4.11	2.91	8.67	0.97	4.99	339
18	0.84	Low (0.19%)	3.09	0.11	0.06	0.56	0.04	0.02	0.06	0.07	0.16	0.46	142
19	0.42	Induced (3x)	0.93	16.47	0.01	0.43	2.28	0.05	0.08	0.02	0.04	2.40	69
20	0.80	Induced (11x)	0.39	0.28	9.36	6.44	12.37	10.14	12.71	1.85	14.30	19.54	132
21	0.51	Low (0.14%)	0.20	0.48	5.82	6.45	3.08	13.83	4.09	1.72	4.08	0.30	149
22*	1.17	Low (0.2%)	1.00	4.27	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1682
24	1.12	n.d.	n.d.	n.d.	0.30	1.65	0.60	0.80	0.12	2.84	0.14	2.81	504
25	1.07	High (0.92%)	1.83	0.27	0.47	12.02	0.005	0.48	0.53	0.62	3.32	0.01	147
26†	1.36	Low (0.61%)	0.90	0.35	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	99
Mean			1.39	2.54	4.69	3.18	5.06	3.83	2.05	1.69	2.30	6.95	294.29
Median			1.22	0.71	0.97	1.81	1.02	0.67	0.49	1.56	0.96	2.40	144.00

Table 18: Summary of the presence of immune modulatory effects in the individual patients. Blue highlights indicate potential negative effects on vaccine efficacy. Missing values in the WT1-panel derive from limited material stocks. Blanks in the immunodulatory panel are attributed to the differential clinical outcome, as not all patients went into relapse, therefore not beeing available for blast analyses. * the patient is still in SD. † the patient relapsed but could not be analysed due to sample limitations.

The different blast-associated immune modulatory mechanisms are hetereogenously distributed among the patients and the presence of elevated levels of the same can be observed in 13 out of 15 patients at time point of relapse. Additionally, 15 out of 17 patients exhibit elevated T_{reg} or MDSC levels as well as restrained CTL-functionality.

We appointed to each of these effects a score value and plotted the total, accumulated amount of each patient versus the clinical outcome in terms of PFS. As seen in Figure 33, the accumulation of multiple immune modulatory parameters can asymptotically be related to a shorter progression-free-survival. Patients with the accumulation of multiple „detrimental“ factors therefor exhibit a shorter PFS than patients with only few immune interfering factors as outlined in Table 19.

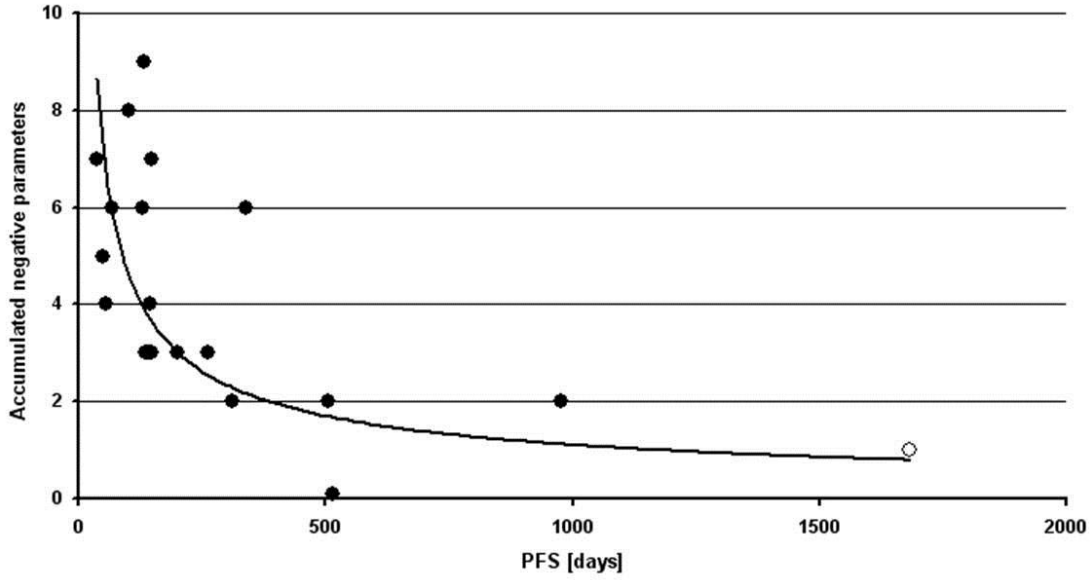


Figure 33: Correlation of the multiplicity of aquired immune modulative mechanisms and PFS. Patient 26 was excluded in this graph, as the missing analysis of blast-associated immune evasive mechanisms could not be performed. ° the patient is still in SD.

Unfortunately, the low number of patients included in our study does not allow to proove this statistically. Nevertheless, this trend should be taken into account when optimizing future treatments.

Total	N	PFS-Range [days]	PFS-mean [days]
0	1	514	514
1	1	1682	1682
2	3	142 - 978	766
3	4	136 - 201	287.75
4	2	57 – 147	102
5	4	49 – 144	97.5
7	1	339	339
8	2	37 – 149	93
9	1	101	101
10	1	132	132

Table 19: Accumulation of immune modulatory mechanisms in patients and association to PFS. The accumulation of immune evasive mechanisms leads to a net reduction of progression free-survival.

4.6 Generation and expansion of WT1-specific T cells

As noted, the recognition of altered cells by the immune system is a crucial step in tumor eradication. The low-affinity-level between TCR and TAA may produce a TAA-specific antigen-threshold below which T cells are not fully activated. In the context of $WT1_{128-134,}$ detailed analyses focusing on the lytic capacity of $WT1_{128-134}$-specific T cells towards leukemic blasts are missing.

This is mainly attributed to the fact, that, up to date, no stable expansion of WT1-specific T cells could be established. Despite the presence and induction of $WT1_{128-}$

$_{134}$-specific T cells in peripheral blood and bone marrow of vaccinated patients, it reamains very difficult to expand WT1-specific T cells *in vivo*. As it appears that succesfull expansion of WT1-specific T cells depnds upon cell culture conditions [266], several protocols were tested for optimization of T cell expansion (Table 20) during this work.

Protocol	Cytokines	Details	Processing	Outcome
1	IL-2 / IL-7	Cells are primed with peptide and IL-2 (50 IU/ml) / IL-7 (2400IU(=10 ng/ml)	Expansion for 10 days, every 2nd day addition of IL-2, every 7th day IL-7	Effective growth, numerous colonies. Low tetramer-frequencies (up to 2 %). Poor cytokine response. No killing.
2	IL-2 / IL-4 / IL-7	Cells are primed with peptide and IL-4 (5 ng/ml) / IL-7 (5 ng/ml)	Expansion for 10 days, every 2nd day addition of IL-2 and IL-4	No functional difference to the SOP. Slightly increased killing if compared to SOP.
3	IL-2	Cell-sorting and incubation with feeder cells and 120 U IL-2.	Every 2nd day addition of IL-2.	Numerous colonies, but no specificity, no killing. (probably due to mal-sorting)
4	IL-7 / IL-12 / IL-2	Cells are primed with peptide.	On day 1 addition of IL-7 (20 ng/ml) and IL-12 (0.5 ng/ml). Then every 2nd day addition of IL-2 (30IU/ml). Weekly restimulation with irradiated and primed PBMCs.	Poor cell growth, isolated colonies. Low tetramer-frequencies (up to 1, 2 %). Mainly TNF-α-producers. No killing
5	IL-7 / IL-10 / IL-12 / IL-2	Cells are primed with peptide.	On day 1 addition of IL-7 (20 ng/ml), IL-10 (10 ng/ml) and IL-12 (0.1 ng/ml), Then every 2nd day addition of IL-2 (30IU/ml). Weekly restimulation with irradiated and primed PBMCs.	Effective growth, numerous colonies. Low tetramer-frequencies (0, 87 %). Mainly TNF-α-producers. No killing
6	IL-6 / IL-12 / IL-2	Cells are primed with peptide.	On day 1 addition of IL-6 (10 ng/ml) and IL-12 (5 ng/ml). Then every 2nd day addition of IL-2 (30 IU/ml). Weekly restimulation with irradiated and primed PBMCs.	Effective growth, numerous colonies. High tetramer-frequencies (8, 37 %). Mainly TNF-α-producers. No killing
7	IL-7 / IL-15 / IL-2	Cells are primed with peptide.	On day 1 addition of IL-7 (20 ng/ml), on day 5 addition of IL-15 (5 ng/ml). Then every 2nd day addition of IL-2 (30IU/ml). Weekly restimulation with irradiated and primed PBMCs.	Poor cell growth, isolated colonies. Low tetramer-frequencies (max 0, 6%). Mainly TNF-α-producers. No killing
8	IL-6 / IL-12 / IL-2 / IL-7	Cells are primed with peptide.	On day 1 addition of IL-6 (10 ng/ml) and IL-12 (5 ng/ml). Then every 2nd day addition of IL-2 (30 IU/ml) and every 7th day IL-7 (10 ng/ml).Weekly restimulation with irradiated and primed PBMCs	Effective growth, numerous colonies. High tetramer-frequencies (constant rates of 5%). Mainly TNF-α-producers.

Table 20: Listing of the applied expansion protocols during this work

In cooperation with the group of Per Thor Straten in Herlev, Denmark PBMCs of two patients (P22 and P12) with high WT1$_{128-134}$-Tetramer frequencies were thawed and prepared for a live experiment on FACSDiva, *i.e.* stained with fluorochrome-conjugated antibodies to CD3 (FITC), CD8 (PE) and with fluorochrome-conjugated WT1$_{128-134}$-Tetramers (APC) (Protocol 3.).

Small lymphocytes were gated by FSC and SSC and further discriminated by CD3, CD8 and WT1$_{128-134}$-Tetramer gating. CD8 / WT1$_{128-134}$-Tetramer double positives were sorted as single cells into 96 well plates, containing approx. 10^5 feeder-cells per well together with phytohemagglutinin (PHA) and 120 U/ml IL-2. Plates were

incubated and medium was changed every 3-4 days. Following 10 – 14 days of incubation, growing clones were transferred into new 96 well plates together with feeder cells and further expanded.

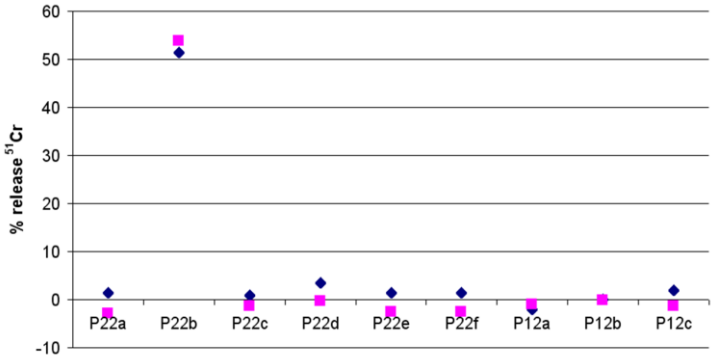


Figure 34: ^{51}Chromium-release assay to detect WT1-specific clones upon FACS selction. Growing clones of two patients were tested in a ^{51}Chromium-release assay, to determine the selction specifity of the performed cell sorting. pink = specific lysis, purple = unspecific lysis.

A total of seven outgrowing clones were selected for patient 22 and a total of three clones for patient 12, respectively. Unlikely no specific $WT1_{128-134}$ specific cytotoxicity, exhibited by the selected clones could be detected by a ^{51}Chromium-release Assay (Figure 34).

Cytokine	Potential effect
IL-2	Stimulates growth, differentiation and survival of CTLs
IL-4	Enhances T cell proliferation
IL-6	Induces a transcriptional inflammatory response (JAK-STAT/MAP-Kinase)
IL-7	Promotes T cell survival and development
IL-10	Stimulates T cells
IL-12	Enhances cytotoxic activity of CTLs
IL-15	Stimulates cell proliferation and activation

Table 21: Activities of the cytokines included in the different expansion protocols.

This was in concert with the analogously performed $WT1_{128-134}$-Tetramer staining, which also proofed poor specificity of the corresponding clones. As direct selection of $WT1_{128-134}$-Tetramer-positive T cells from PBMCs seemed to be inadequate for efficient and stable expansion of these cells, the experimental setting was altered to an initial stimulation of PBMCs with $WT1_{128-134}$ and consecutive incubation with different expansion cocktails (protocols 4 - 8).

In cooperation with Dr. Agnieszka Wieczorek and Prof. Dr. Lutz Uharek, different expansion protocols, based on different cytokine-cocktails were tested (Table 20).

Experiments performed with samples from three different patients revealed that protocol 4, based on IL-6 and IL-12 initially resulted in sufficient amounts of WT1-specific T cells. The consecutive modification of the post-stimulation processing by

adding IL-7 (protocol 8) resulted in a stable selection of WT1-specific T cells, oscillating at a frequency of 5% and seems to be the most efficient protocol.

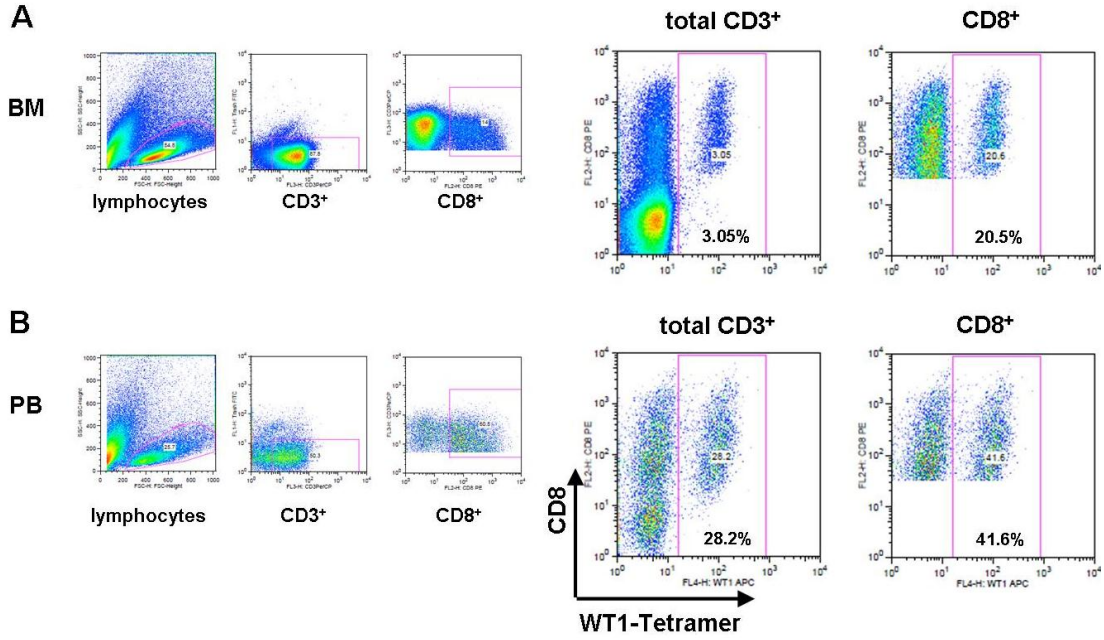


Figure 35: WT1-specificity of expanded T cells in BM and PB. T cells were expanded using $WT1_{126-134}$-peptide together with IL-6 and IL-12 for initial boosting, as well as IL-2 and IL-7 for consecutive maintenance. **A)** Bone marrow-derived lymphocytes exhibit WT1-specific frequencies of 3% in the total CD3+ fraction and 20% in the CD3+CD8+ fraction, respectively. **B)** PBMC-derived lymphocytes exhibit WT1-specific frequencies of 28% in the total CD3+ fraction and 41% in the CD3+CD8+ fraction, respectively. A control staining of the PBMC sample with an HIV-Tetramer excluded unspecific staining of the corresponding WT1-tetramers (data not shown).

We further modified the protocol, and by combination with the MACS-technique, we succeeded in expanding WT1-specific T cells in frequencies up to 40% of PB-derived $CD3^+CD8^+$ T cells (Figure 35).To further characterize these expanded WT1-specific T cells, a RT PCR-based relative *Vβ*-family quantification approach [267], established in an associated lab, was performed.

After separation of WT1-$Tetramer^+$-T cells from their negative counterparts by MACS, both subsets were screened for *Vβ* -chain family expression. The subsets differ significantly in their *Vβ* -chain family phenotype (Figure 36). The WT1-$Tetramer^-$ fraction, composed of unprimed irradiated PBMCs, exhibits a variation of different *Vβ* –chains, with focus on *Vβ* 2, *Vβ* 6a and *Vβ* 8. In contrast, the isolated WT1-$Tetramer^+$-T cell fraction is mainly composed out of cells expressing *Vβ* 7 (up to 90 %).

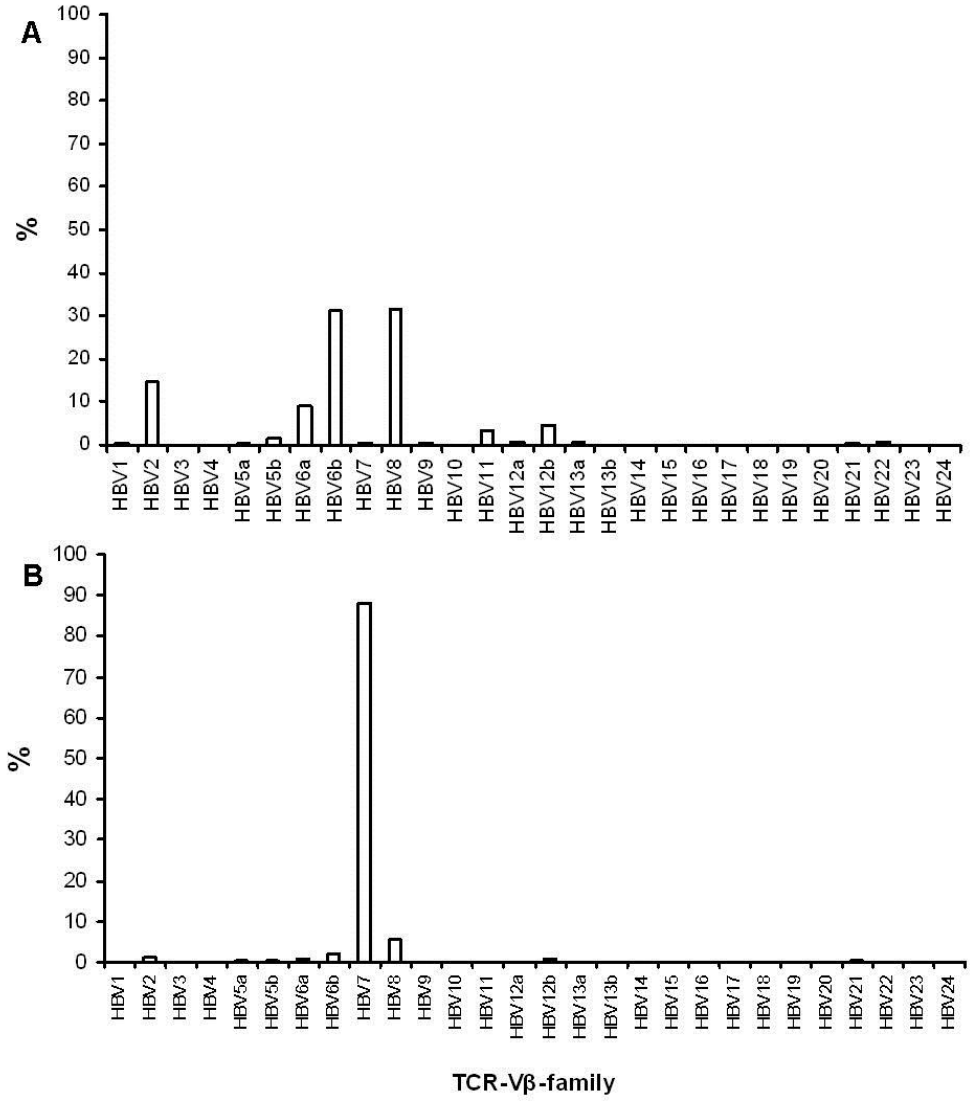


Figure 36: Distribution of TCR- *Vβ* -chain families in the differential subsets of expanded T cells. A) WT1-Tet^{neg} cells do exhibit a broad and heterogeneous range of different TCR- *Vβ* –chain families. B) In contrast, the MACS-sorted WT1-Tet^{pos} cells mainly express the TCR- *Vβ* –chain family HBV7 (~90%) suggesting monoclonality of the selected WT1-specific T cells.

4.7 Tumor recognition and eradication

In preparation for experiments testing the ability of WT1-specific T cells to recognize and eradicate autologous and allogeneic leukeimic cells suitable assays were established during this work.

As a proof of principle, we screened several leukemic cell lines for their suitability as target cells for the detection of WT1-specific cytokine production and cytotoxicity, respectively. We also screened the available leukemic cell lines on their expression profiles on HLA-A2 and WT1. Out of 6 leukemic cell lines 4 turned out to be HLA-A2 positive (Figure 37 A). The HLA-A2 negative cell line Molt-4 was subsequently used as a negative control in all peptide loading experiments.

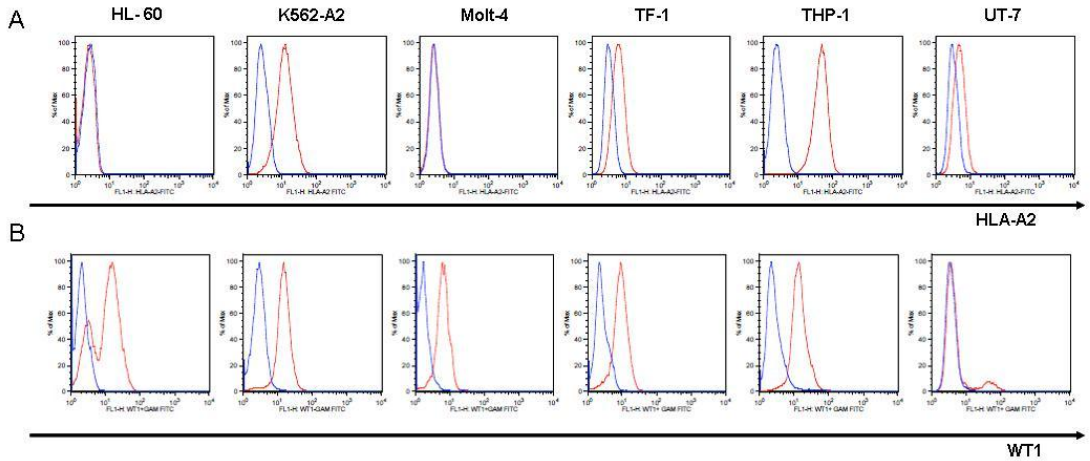


Figure 37: Expression profiles of available leukemic cell lines. A) Surface staining of different tumor cell lines with anti-HLA-A2. Except for HL-60 and Molt-4, all remaining cell lines present HLA-A2 on their surface. **B)** Intranuclear staining of the same tumor cell lines with anti-WT1. All cell lines express the transcription factor WT1. Interestingly, in the case if UT-7, only a small proportion of cells seems to be $WT1^+$.

In the case of WT1-expression, 5 cell lines were significantly positive for the transcription factor, whereas the cell line UT-7 exhibited only a negligible amount of WT1-positivity (Figure 37 B).

In a next step, we incubated four HLA-A2 positive cell lines (K562-A2, THP-1, TF-1 and UT-7) as well as one HLA-A2 negative cell line (Molt-4) with an HLA-A2-restricted Influenza A-peptide or an irrelevant HIV-peptide. When cultured over-night together with $HLA\text{-}A2^+$ T cells, expanded for 14 d after priming with the same Influenza A-peptide, specific secretion of IFN-γ and TNF-α could determined by flow-cytometry. No specific cytokine production of Influenza A-specific T cells could be observed when incubated together with the HLA-A2 negative cell line Molt 4 (Figure 38 A). In contrast, all four HLA-A2 positive cell lines were able to specifically activate the expanded T cells (Figure 38 B).

In addition to the analyses of WT1-dependent cytokine production we were looking for suitable assay to directly determine WT1-specific cytotoxity. The first attempts were designed according to publications by Schmid *et al.* [268], Lecoeur *et al.*[269] and Fischer *et al.*[270] , using fluorescent dyes for live/dead discrimination. In brief, target and effector cells were stained with an exclusion marker (CFDA-SE or PKH-26), the target cells were loaded with the antigenic peptide and coincubation was performed for 24 h. Cytotoxicity was the determined by amount of $AnnexinV^+$-cells in the target population.

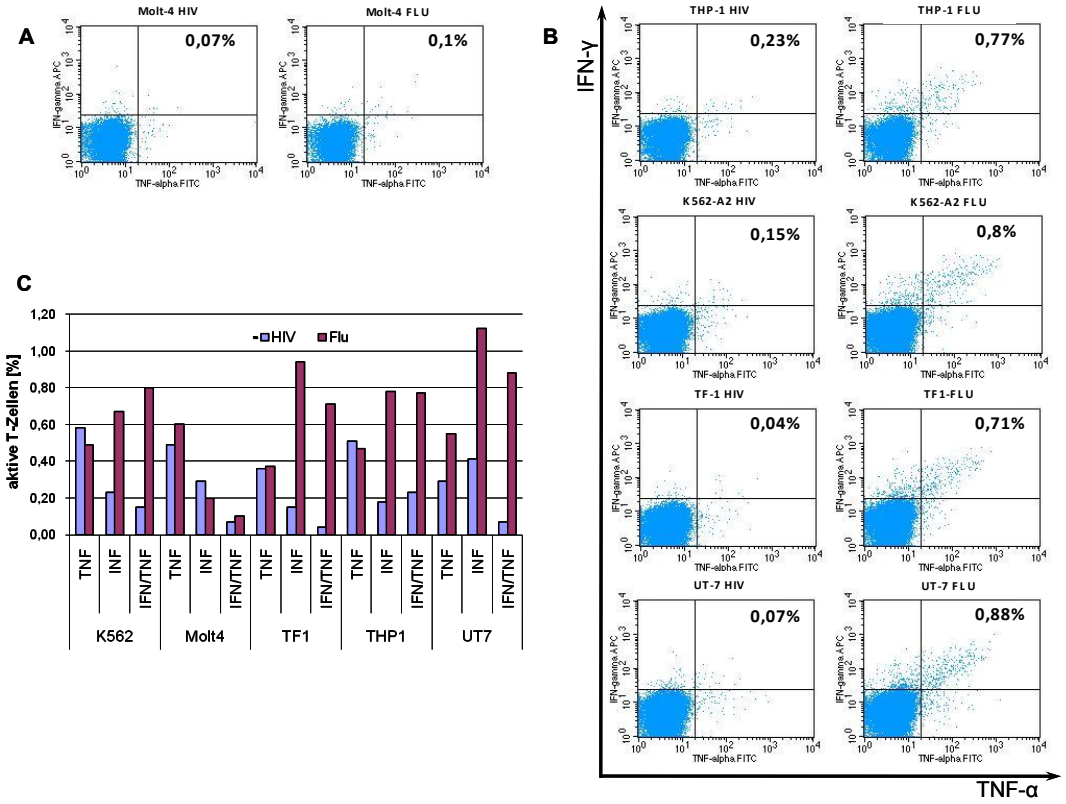


Figure 38: Characterization of leukemic cell lines in order to specifically activate antigen-primed T cells. Influenza A- primed PBMCs from an HLA-A2^{+}Influenza^{+}-donor were co-incubated with different leukemic cell lines, loaded with HLA-A2-restricted Infuenza A (FLU)- or an irrelevant peptide (HIV) and screened for cytokine release. **A)** The HLA-A2 negative cell line Molt-4 is not able to specifically activate Influenza A-primed T cells. **B)** In contrast, all 4 HLA-A2 positive cell lines activate Influenza A-primed T cells as can be observed by the enhanced cytokine release in the Influenza A-loaded setting. **C)** Column bar graph, indicating the specific activation of primed PBMCs in the different leukemic cell lines in regards to the stimulation with both peptides. In contrast to the negative control Molt-4, all other cell lines specifically activate Influenza-primed PBMCs as observed by an at least 2-fold increase of cytokine release in response to FLU-loaded cells in comparison to HIV-loaded cells.

These assays perform quite effective when dealing with sufficient cell numbers and T cell specifities. As we had to notice that the expansion of WT1-specific T cells usually leads to low frequencies of usable effector-cells, we started to screen for a more sensitive assay. In cooperation with the group of Dr. A.M. Kaufmann we established a cytotox-assay for our needs[244].

The VITAL-FR assay, which has been published in 2004 by Hermans *et al.* [271], combines specific and unspecific killing in one experiment. Target cells are differentially labeled with fluorescent dyes and loaded with a) the target peptide or b) a control peptide. As the differentially loaded cells can be attributed to specific colors, lysis can be determined flow cytometrically by the ratio of the remaining viable control and target cells (Figure 39 B).

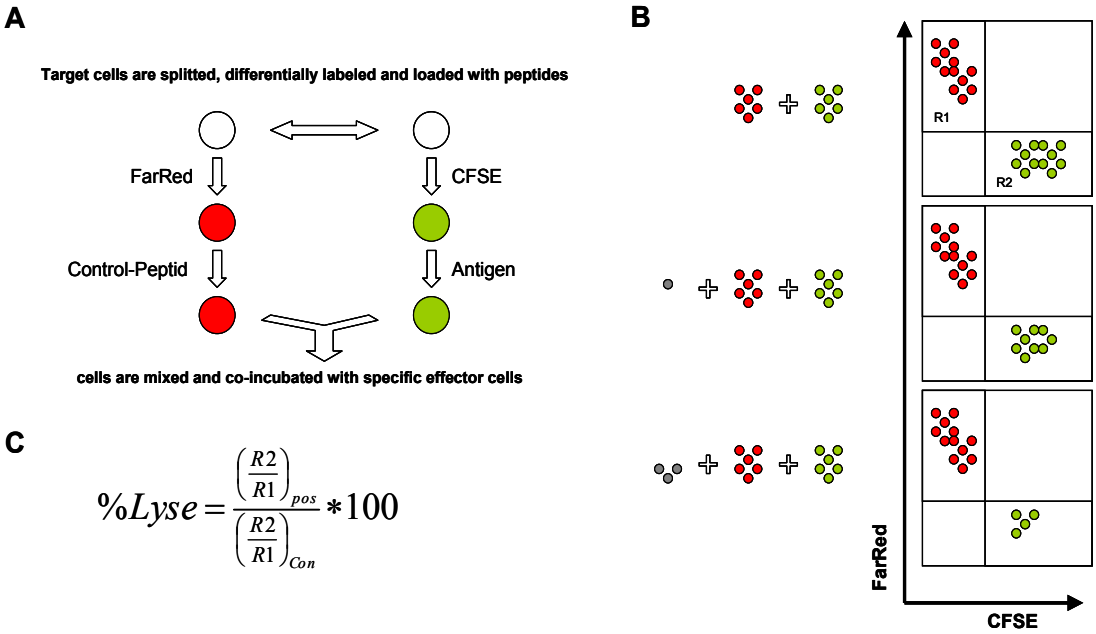


Figure 39: Methodical setting of the VITAL-FR assay to determine specific cytotoxicity. A) Preparations for the VITAL-FR assay. The target cell line is splitted and differentially labeled with fluorescent dyes. After labeling, the two subpopulations are either loaded with the specific antigen or a control peptide. **B)** Schematic experimental illustration: With increasing amount of effector cells (grey), the amount of antigen-loaded cells is decreasing, thus affecting the ratio between the two cellular subsets. **C)** Equation leading to the net percentage of lysed cells.

Under optimal conditions the VITAL-FR assay is supposed to be able to significantly detect cytotoxic effects induced by three specific CTL[244]. In a first attempts to determine if this assay is suitable in our leukemic context we performed an experiment in which differentially labeled UT-7 cells were loaded with either an HLA-A2-restricted Influenza A- or HIV-peptide. These cells were consecutively co-cultured with Influenza A-primed T cells at different E.T ratios overnight and analysed flow-cytometrically (Figure 40 A).

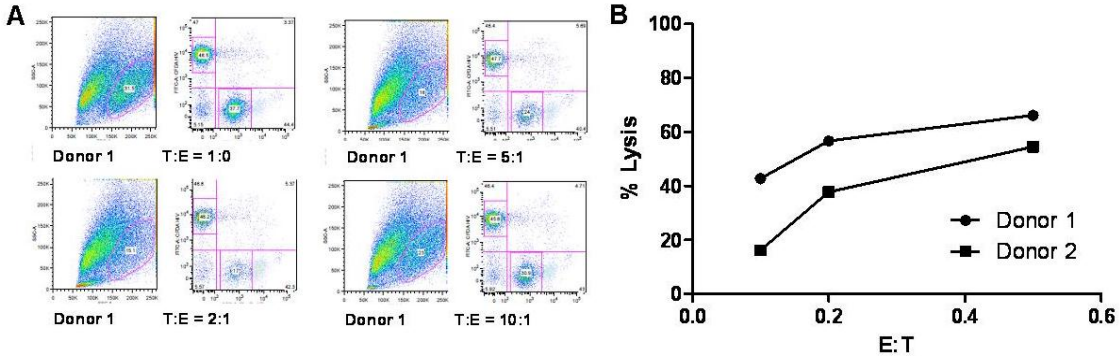


Figure 40: Preliminary testing of the VITAL-FR assay in a leukimc context. A) Flow-cytometric analysis of an experiment with leukocytic material of donor 1. After initial exclusion of the lymphocyte population, the tumor cells are displayed according to their initial labeling with FarRed or CFSE, implicating the loading with Influenza A- or HIV-peptide. **B)** The comparison of the different fluorochrome ratios in the context of increasing E:T rations leads to lytic saturation curve for both experimental approaches.

The experiment was performed in duplicate, using two different Influenaza-reactive donors. The corresponding graph (Figure 40 B) clearly shows the positive lytic capacity of both T cell populations, thereby proofing the suitability of the VITAL-FR assay in a leukemic context.

5 Discussion

This work describes the monitoring of humoral and cellular immune responses in patients with AML in the context of a peptide-based anti-cancer vaccine and the potential complex immune-restrictions caused by functionally impaired tumor-specific T cells or immune evading leukemic blast interfering with vaccine efficacy.
Prior to vaccination all patients had active AML or were at high risk of early relapse after chemotherapy. In brief, the vaccination with WT1 peptide together with KLH and GM-CSF as adjuvants [93] demonstrated unexpected clinical efficacy by stabilization of the disease in a substantial number of patients with active AML and delayed relapses in half of the high-risk patients. The clinical responses was correlated to the molecular efficacy with 52% of patients, in which WT1-mRNA levels in PB decreased to at least one third compared to baseline. The number of patients with induced $WT1_{128\text{-}134}$-specific T-cells increased from 28% at baseline to 76% in week 10 demonstrating immunological efficacy, which was however not correlated to molecular or clinical efficacy of WT1-peptide vaccination. Despite these encouraging results most of the patients relapse after a certain time, which made an additional detailed immunomonitoring and the analyses of potential immune escape mechanism necessary.

5.1 Evaluation of humoral reactivity to the vaccination with WT-1-peptide plus GM-CSF and KLH

In the context of tumor eradication, as well as in many other pathological conditions, the humoral part of the immune system plays an important role, as demonstrated by several clinical therapies using directed antibodies to mark tumor cells and facilitate tumor recognition [31, 272] and destruction.
In the case of the tumor antigen WT1, several studies have described the presence of spontaneous natural humoral immune responses against WT1 in patients with WT1-expressing hematopoietic malignancies in *vitro* and *in vivo* [124]. Based on this data, we analyzed the WT1 specific humoral response induced by the administration of an HLA-A201 restricted epitope of WT1 ($WT1_{126–134}$) in combination with KLH and GM-CSF. Additionally, we evaluated if the induction of WT1-antibodies can also serve as serological marker for vaccine efficacy by analyzing whether the induction

of WT1-specific IgM-antibodies was associated with the clinical outcome and survival of the patients.

In our specific vaccine setting, we were able to demonstrate an induction of WT1-specific IgM-antibodies but not antibodies with an IgG-subtype in response to consecutive treatment. As the majority of initial IgM-responses was detected around week 26/30 (5 out of 7 patients) we started to investigate if the lack of isotype-switch was due to time reasons or a lack of specific B cell/T-helper cell interaction. This is of special concern, as in our specific setting the immunogenic part of vaccine is composed of a MHC-class I-restricted epitope, intentionally designed to activate $CD8^+$ T cells. In contrast to the induction of M-isotype immunoglobulins, the secretion of G-isotype immunoglobulins requires the transformation of a B cell into a plasma cell, a process which is initiated upon CD40 stimulation through $CD4^+$ T-helper cell interaction. Indeed, further experiments revealed that in our study we were unable to induce WT1- specific $CD4^+$ cells. One can further speculate that the lysis of leukemic blasts by vaccine-induced CTLs seems to be insufficient to provide further "natural" WT1 epitopes for alternative T-helper cell stimulation.

Concerning the suitability of WT1 antibodies as serological marker, we could observe a significant number of patients with a favourable clinical course (SD or remission) who also had elevated titers of WT1-specific IgM antibodies, compared to those who did not respond clinically to the WT1 vaccination therapy (PD or relapse).

Our data is in line with most other HLA-Class-I-epitope-based vaccines, which generally show poor ability to induce balanced activation of $CD4^+$ - and $CD8^+$ -T cell subsets, which might be essential for effective, long-lasting antitumor immunity. Indeed, a current publication describes HLA-A2-restricted responses that efficiently kill tumor cells but are characterized by a limited lifespan in the absence of $CD4^+$ helper T cells [231].

Data from Nicoli *et al.* suggest that natural induction and isotype-switching of WT1-specific immunoglobulins depends on strong and persistent stimulation with WT1 as only seen in patients with high-blast count [273], but not in healthy subjects. As WT1 seems already to be a naturally weak B-cell stimulus, which is in line with the

reported absence of toxicity and autoimmune phenomena after WT1 vaccination [133], the administration of only an HLA-A201-restricted epitope seems to be detrimental to the conversion of WT1-specific IgM to IgG.

These conclusions are supported by the fact that the administration of the "non-self"-protein KLH as immunostimulant resulted in the induction of both, IgM- and IgG-antibodies, specific for KLH, proofing an otherwise intact conversion system. In contrast to WT1, KLH was administered as an intact protein and could be processed in APCs to deliver both, MHC-class I and MHC-class II restricted epitopes. It is known, that protein-based vaccines are capable of generating stronger $CD4^+$ responses, but – unfortunately - at the cost of less effective induction of CTLs [274].

The induction of anti-KLH immunoglobulins precedes the one of anti-WT1 immunoglobulins as observed in different vaccine settings using KLH [92] and is presumably a result of a boost of naturally occurring anti-KLH antibody responses[275]. These data are consistent with the preliminary testing of negative controls. Two healthy donors showed an elevated spontaneous response to KLH, probably due to oral tolerance and were consequentially excluded as controls.

A long-term vaccination with an HLA-A2/MHC class I- restricted peptide is therefore able to induce WT1-specific IgM antibodies. But the failure to induce IgG antibodies gives evidence that specific T-helper-cell induction by the MHC class I peptide, KLH and GM-CSF is lacking for induction of isotype switch of B cells, leading to optimal immune response against WT1. Nevertheless, these results demonstrate that anti-WT1-IgM can be used as serological marker for treatment efficacy.
Most of the remarks described above could be bypassed by the use of longer peptides eligible for intracellular processing, or the combination of several different epitopes in the same vaccine. One possibility to enhance the efficacy of vaccines would be the use of WT1-Pepmixes, comprising the entire WT1 protein, split into overlapping peptides of 15 AA and therefore combining both features, in eliciting long lasting and complete immune responses.

5.2 Evaluation of T cell functionality and anergy

In our previous work, we could demonstrate immunogenicity of our specific vaccine setting, as observed by the induction of TAA-specific T cell responses in 52% of the patients during time-course. Nevertheless, these responses were predominantly short term effector responses and could be transformed into long-lasting memory responses in only a few cases, In addition this work was intended to reveal pontential cellular effects detrimental to vaccine efficacy. The efficacy of cancer immunotherapy strongly relies in addition to external factors, *e.g.* the selection of an appropriate epitope as well as a suitable delivery system, on the individual immunological status of the treated patient. On this account, therapy-related monitoring of T cell functionality as well as periodically screening for potential mechanisms interfering with proper T cell function should represent a necessity in cancer immunotherapy. As the latter field still resides in an investigative state, context-specific markers for appropriate or failing T cell functionality have to be identified during early phase-studies.

While immediate protection is conferred by circulating or tissue-resident effector memory T cells, recall responses are mediated by central memory T cells that patrol the T cell areas of secondary lymphoid organs where they can rapidly proliferate and respond with greater vigor upon re-encounter with the same pathogen [202, 276]. The migration to the SLOs requires the expression of so-called homing-receptors, of which CCR7 is attributed to central memory T cells [199].

The results presented in this work show, that a vaccine-induced increase in CCR7-frequencies in $CD8^+$ T cells is associated with a better prognosis in terms of progression-free survival. The opposite is true for patients with decreased frequencies compared to baseline. These patients exhibit a significantly shorter progression-free survival than patients with increased CCR7 values ($p< 0.01$). As both groups did not inherit significant differences in CCR7 expression at baseline, one can speculate if the variances in CCR7 frequencies are provoked by individual circumstances leading to a beneficial response or if they are the result of a general mechanism modulating the differentiation and the proliferative potential of T cells and/or T cell exhaustion or anergy.

Interestingly, an *addendum* presented at the 2011 ASCO meeting (abstract 2503), referring to a recently published study on the effect of Ipilimumab-treatment in resected melanoma patients [277] by Sarnaik and collegues, equally demonstrated an association of decreased CCR7-frequencies with relapse and/or death in their patient cohort. As Ipilimumab is designed to interfere with the immune checkpoint factor CTLA-4, a molecule triggering T-cell anergy, the consecutive eradication of residual disease and hence the clinical outcome are mainly affected by the effector-capacity of the patients' T cells. Thus individuals with sufficient amounts of $T_{naïve}$ and T_{cm}-cells, *i.e.* CRR7-expressing phenotypes should exhibit a beneficial clinical performance. In accordance with this assumption, Sarnaik *et al.* additionally observed a parasllel increase of eomesdormin-frequencies in T-cells of patients with relapse or death, wich represents an additional indicator for a late maturation status and therefore reduced lifespan of the corresponding T-cells [278].

It is hypothesized, that long-term maintenance of an immunological memory would be dependent on cells that retain their proliferative capacity [279], which is a characteristics of T_{CM} rather than T_{EM} cells [280]. Consecutive encounter with an antigen may result in immunological tolerance towards the antigen in terms of impaired activation or induced anergy of antigen-specific T cells. As in any other immunogenic context, only a small fraction of the tumor-specific cells present at the peak of the immune response survives as memory cells in the peripheral lymphoid organs[281]. Further confrontation with the specific antigen induces recall responses mediated by central memory T cells, which can rapidly proliferate and differentiate into effector memory T cells, displaying immediate effector functions. However, recent studies have shown that repeated boosting, as performed in our vaccination scheme, can drive memory T cells toward terminal differentiation[282, 283] accompanied by reduced or no proliferative and reconstitution capacity of T_{EM}, as well includes the risk of depleting the population of central memory T cells.

On this account, the monitoring of a specific patient in our collective is quite interesting: the patient achieved a complete remission for 12 month and the expanded WT1-specific T cell clones were analysed by qRT PCR quantification for all TCR Vβ-families. A specific predominant clone was identified during clinical remission and was present in PB and BM after 8 vaccinations. During further vaccination

course the transcript levels in BM decreased and at relapse frequencies of the specific clone raised only in PB but not in BM [284]. These data, support the present findings on impaired memory function as well provide first data regarding evolution and compartmentalization of a peptide vaccine-induced T cell clone in PB and BM.

As a part of this work was intended to investigate potential effects of T-cell exhaustion we were able to demonstrate that, in fact, vaccine efficacy also is strongly determined by the actual exhaustion status of the lymphocyte population at therapy initiation.
The results show that increased PD-1 values at baseline could be associated to a shorter PFS (median =132 days; p=0.05) in contrast to patients with initially low PD-1 frequencies. In addition, these subsets not only differed in terms of PFS but also in terms of a molecular response towards the vaccine. Low PD-1 values at baseline were clearly associated (p=0.057) with a functional molecular response, as observed by a net reduction of WT1-mRNA levels in week 10.
Notably, the major knowledge about the PD-1/PD-L1 pathway derives from murine tumor models [160, 285, 286]. On this account and despite the xenogeneic differences, our data is in concordance with two systemic models of murine AML established in the USA [16, 287]. Both models proofed increased resistance to AML in PD-1(-/-) knockout mice in contrast to wild-type-mice and additionally proofed increased function of CTLs at tumor sites upon PD-1/PD-L1 blockade. These effects support the assumption that increased PD-1 frequencies at an early stage of immunotherapy may be crucial for long-term PFS.

Taken together, the data on T-cell functionality suggest that consecutive vaccination may lead to reduced proliferative capacity of T cells, interfering with a proper induction of an immunologic memory. Further, our data suggests, elevated PD-1 expression, prior to vaccination or acquired, is crucial for long-term relapse-free survival. In contrast, constantly low or reduction of PD-1 seems to account for favorable prognosis, an effect close to observations in chronic viral infections [288].).
The same outcome holds true in regards of the PD-1 ligand PD-L1 on leukemic blasts: AML patients in which blasts increase PD-L1 expression, tend to relapse earlier, than do the low-PD-L1 counterparts.

5.3 Impact of escape mechanisms of leukemic blasts on vaccine efficacy

In addition to an inert immunologic tolerance or poor response towards the tumor, tumor progression may also be facilitated by the induction of suppressive effects or escape phenomena by the tumor itself (*c.f.* section 1.4). The hematopoietic descendance of leukemias leads to an exclusive situation, as the two opponing forces, the leukemic blasts as well as the targeting immune cells, potentially revert to a similar transkriptome[14] and/or signalome[13]. On this account the deciphering of potential immunomodulatory effects of the leukemic blasts towards the immune response represents a clinically importance as well as scientifically interesting aspect of immune monitoring in AML and other leukemias.

Along with stimulatory modulators like TGF-β and CXCR4, that act ubiquitously on most cell-types and therefore are frequently found in a broad range of different neoplasias, this work also focused on the role of negative factors that naturally appear in an immunologic context. In fact, investigations on PI-9, IDO, IL-10, HMOX and PDL1, that play a regulative role in immune privilege, peripheral tolerance and/or other immunomodulatory pathways, comprised the largest component regarding this section of the presented work.

In our specific setting, these blast-associated modulators were hetereogenously distributed among the patients but could not significantly be associated to a general therapeutic effect. Nevertheless, their presence and induction in individual cases surely may influence the individual outcome of single patients.

In coincidence with the gathered PD-1 data, PD-L1/CD274 showed a significantly altered expression during vaccination which could be correlated to a higher PFS (p= *; Figure 32 A) in affected patients. Despite a diversified range of individual PD-L1 expression levels among different patients and tumor entities when compared to healthy controls, the median values of PD-L1-mRNA levels seem to be on a similar level with those of healthy controls. However, in our specific setting, a fraction of our patients exhibited an elevated median level of PD-L1 mRNA at baseline which

significantly declined during therapy. Interestingly, these exclusively were patients who benefitted the most from the vaccination and had longer PFS.

The explanations are speculative, but it may be possible, that a conjuncture of the differentiation status of the tumor progenitor cell as well as the *de novo* induction of "non-exhausted" WT1-specific T cells may lead to such an effect. Indeed, it has been recently described that PD-L1 expression differs on hematopoietic progenitor cells, according to the maturation status and the environmental inflammatory conditions [289] in patients after HSCT. Additionally, in a murine model of AML, Zhang *et al* observed that PD-L1-expression is elevated on murine C1498 blasts growing in an *in vivo* context when compared to the same cells cultured *in vitro* [16], but that external administration of IFN-y to the *in vitro* culture leads to a leveling of both expression patterns.
These data points to the general importance of the PD-1/PD-L1 pathway in immune evasion by a hematologic malignancy, but also provides additional information on individual parameters enhancing this effect. Nevertheless, these data equally supports the necessity of current and future clinical trials targeting this pathway in leukemia patients.

When comparing our patients to healthy controls and unvaccinated AML-patients we also observe that AML-patients in general seem to have lower TGF-β values than have healthy controls. This effect may be a molecular response to reduced TGF-β signaling in AML caused by functional change of SMAD4, as described earlier [290].
When retaining the previous discrimination in patients with short and long PFS, TGF-β-mRNA levels perform oppositely: While TGF-β ratios are reduced in the short-PFS subset, TGF-β values increase in the patients with longer PFS (Figure 32 B). In theory, when the patients relapse, it is assumed that TGF-β might act as a tumor-promoter. It appears paradox that a reduced TGF-β-expression should lead to an increased tumor progression, while augmented TGF-β-expression delays tumor spreading. But this effect might be explained by the bifurcated downstream events in case of loss of TGF-β signaling[17]:
As mentioned earlier, transforming-growth-factor-β plays an important role in tumor initiation and progression, functioning as both a suppressor and a promoter. TGF- β normally supports homeostasis and suppresses tumor progression. However, a break

in its pathway triggers tumor growth and metastasis. Mutations of the genes encoding TβRI and TβRII, or decreased expression and phosphorylation of other components of this pathway, have been reported in human cancers [17].

TGF-β suppresses tumor initiation and early development through the inhibition of cell cycle progression, induction of apoptosis, and suppression of growth factor, cytokine and chemokine expression. Relying on the kind of TGF-β–signaling disruption, tumor initiation and promotion occur differentially. Impairment in the early phase of the TGF-β signal cascade, *e.g.* loss or mutation of signaling components, such as TGF-β-receptors, results in rapid growth, *i.e.* increased mitosis rates and therefore increased probability of further cancer-driving mutations, and rapid tumor progression. In contrast, perturbations in the downstream process lead to an either slow expanding tumor but make it more invasive and more likely to metastasize [17]. As we observed different timeframes for tumor relapses, further analyzes should focus on identifying the specific casualties responsible for the TGF-β variances.

5.4 Evaluation of immune regulatory cells

Functional impairment of tumor-specific T cells not always is a result of classical exhaustion and/or modulation by tumor cells: There also exist naturally occurring regulatory cells, intended to avoid collateral damage caused by over-reactive or obsolete cellular immune reactions. These cells, in this work we only concentrate on MDSCs and T_{regs}, represent an additional crucial feature for vaccine efficay that either may be induced naturally or may be provoked by the tumor to serve as interference to anti-tumor immunity.

MDSC are one of the major factors responsible for immune suppression in cancer. They also contribute to limited efficacy of current vaccination strategies. As seen in different clinical settings, the amount of MDSC is elevated in our AML patients compared to healthy controls. Despite the detection of an MDSC-decrease during vaccination, the latter effect could not be proven to be statistically significant associated with favorable clinical course. Nevertheless, the increased frequencies of MDSC point to a potentially restricted immune response in individual patients.

An interesting aspect concerning this subject is the consecutive administration of GM-CSF as part of our vaccination regimen. Physiologic concentrations of GM-CSF

are required for normal myelopoeisis and DC differentiation. However, excessive amounts of this growth factor could exert immune suppressive effects and could be responsible for induction of MDSC as shown by twot studies, in which the administration of GM-CSF resulted in significantly increased numbers of MDSCs [291, 292]. Filipazzi *et al.*analyzed MDSC modulation by a HSPPC-96/GM-CSF vaccine in stage IV melanoma patients. When compared to stage-matched melanoma patients who received HSPPC-96 alone, $CD14^{+}HLA^{-}DR^{-/lo}$ MDSC-frequencies and TGF-β plasma levels were augmented after administration of the GM-CSF-based vaccine [293]. This led to the assumption that GM-CSF-based vaccinations *per se* may be able to potentially expand immune suppressive MDSCs.
In fact, a second study by Solito *et al.* showed that promyelocyte-like BM-MDSCs can be generated from human BM-cells after treatment with a combination of G-CSF and GM-CSF [292]. Additionally, these cells excerted immunosuppressive functions in presence of lymphocytes and were considered equivalent to MDSC in the blood of breast and colorectal cancer patients.

Similar results were obtained consideringr the T_{reg} compartment. Regulatory T cells mediate amelioration of disease and immune homeostasis by inhibiting immune activation and maintaining peripheral immune tolerance. The suppressive mechanisms and clinical significance of Treg have not been completely uncovered in patients with acute myeloid leukemia, but several reports have shown that increased frequencies of circulating $CD4^{+}CD25^{high}$ T_{reg} in patients with AML provide suppressive activity compared with those from healthy controls [294-296].
In our patients, at baseline, we were able to detect elevated levels of $CD4^{+}CD25^{high}$ $CD127^{low}$ when compared to healthy controls. When the T_{reg}-frequencies at baseline were aligned with those in week 10, we pictured a quite heterogeneous development. We could monitor increase as well as reduction of T_{reg}-frequencies in different patients, as well as stable T_{reg}-frequencies in a few patients. Unfortunately these effects could not be correlated to clinical outcome or PFS. Similar effects have been previously described in the context of immunotherapy [297], probably due to a secondary response to therapy-induced inflammation as equally observed in chemotherapeutic settings [296].
It has been shown, that tumor-derived MDSC induce tolerance by expanding naturally occurring tumor-specific T_{reg} [231, 265, 298], but mainly in murine systems.

There exist only few data concerning this effect in human settings, but data is controversial. However, we could not find any indication in our patient population that MDSC and T_{reg} cells might be linked in a common immunoregulatory network that involves both myeloid and T cells, as proposed by others. The monitoring of our vaccine trial showed that the percentage of T_{reg} cells was invariably high throughout tumor growth but did not relate to the kinetics of accumulation of the MDSC population. This reliance could also be observed by Ko *et al.* [299] and Movahedi *et al.* [300], with the latter suggesting that MDSCs are not necessarily required for Treg induction and maturation. Conclusive evidence supporting or rejecting such interaction in humans needs to be satisfactorily confirmed in further studies.

5.5 Expansion of WT1-specific T cells

The stable detection, enumeration and *in vitro*-expansion of antigen-specific T cells are of major importance in T cell immunology and its application in targeted therapy. A detailed characterization of antigen-specific T cells leads to multiple potential applications in cancer therapy, *e.g.* treatment with *in vitro* expanded TAA-specific T cells and/or the generation of TCR-gene-modified T lymphocytes for adoptive T cell therapy, respectively.

On this account and depending on the application, minimum quantities of TAA-specific T cells have to be sampled, expanded and preserved for further processing.

In the context of WT1, strategies to expand specific CTLs have been proofed to be labourous and rarely result in satisfactory amounts of WT1-specific T cells. Investigations on *in vitro* methods to expand WT1-specific T cells in patients and healthy individuals mainly include DC-stimulation [266] as well as WT1-peptide/pepmix-stimulation [301-303], usually generating frequencies ranging from 1 to 6 % of total $CD3^+CD8^+$ cells.

The establishment of WT1 specific T cells in our institution was preceded by the evaluation and comparison of several protocols. In one patient we were able to expand WT1-specific T cells to frequencies of 21% of D3+CD8+WT1-tetramer+ T cells from a PB-sample and 48% 21% of D3+CD8+WT1-tetramer+ T cells generated from a BM-sample, respectively. To our knowledge, this is the first time that such high frequencies could be reported after expansion of natural WT1-specific T cells by WT1-peptide stimulation.

However, these results represent an exceptional and time consuming work effort by means of repeated restimulation of the expanded T cells with irradiated, epitope-loaded APCs and repeated purification by the MACS technique. Up to date, we were not able to successfully transfer this approach to samples collected from other patients in our trial. Notably, among our patient population, patient 22 uniquely exhibited constantly elevated levels of WT1-tetramer specific T cells including stable frequencies of WT1-tetramer specific-T_{EM}-cells (*c.f.* Figure 26). As T_{EM}- cells exhibit an increased proliferative capacity, these cells might be preferable as basis for the expansion of WT1-tetramer specific T cells. In fact, viral-specific approaches have been shown to be more effective when expanding *ex vivo* memory T cells [304, 305].

Contradictotily, some publications concerning the expansion of WT1-specific CTLs also describe the induction of WT1-specific T cells out of material derived from normal healthy donors [266, 301, 303, 306] as detected by multimer staining. For example, Weber *et al.* demonstrated that HLA-A1- and HLA-A2-restricted WT1-specific CTLs could be generated in a limiting dilution assay from the $CD8^+$ lymphocyte fraction of 4 out of 10 healthy adult donors [303]. These cells exhibited similar functional and lytic tumor reactivity like those equally generated from samples of breast cancer patients (4 out of 9). Ho *et al.* also reported succesfull generation of WT1-specifc T-cells (up to 18% after the 3rd stimulation) out of PBMCs of healthy donors by using a DC-based approach [266]. Surprisingly, the group around M. Stamer even described the serendipitous expansion of WT1-specific CTLs from CMVpp65-stimulated $CD8^+$ cells [307]. Taken together, these data would suggest that WT1 specific T cells represent an easy-to-generate subset of the naïve T cell repertoire, contradicting the experienced difficulties in the *ex vivo* expansion of patient-derived natural WT1-specific CTLs and in establishing a permanent population of WT1-specific T lymphocytes in hematological cancers [93, 100, 135].

Despite first initiatives to harmonize guidelines for HLA-multimer assays[248], the use of non-standardized and non-validated assays is still common. This fact exludes direct comparison of gathered data by different labs [308] and hence makes data potentially questionable. This complex situation and the personal experience on the

pitfalls on the evaluation of multimer-staining data support the need for unbiased and harmonized evaluation criteria.

It was recently shown by our group, that a predominant TCR *β* chain of the *Vβ-21-family* was associated with vaccine induced complete remission in an AML patient (patient 1) in our setting [284]. In a follow-up study, the question was adressed whether this predominant clone could be found in other AML patients vaccinated with WT1 peptide (paper in review). In this context, TCR- *Vβ* analyses of CTLs from patient 22 prooved to include T cells expressing a similar *Vβ-21* TCR phenotype as the one predominantly expressed in patient 1.
Having access to a high frequency of *ex vivo* expanded WT1-specific T cells of patient 22, we investigated if these cells also exhibit a unique phenotype concerning their TCR- family. The obtained data concerning the *Vβ* –chain-families indicated that, in contrast to the *in vivo* population, up to 90 % of the *ex vivo* expanded WT1-specific T cells predominantly expressed the *Vβ-7*-chain. Taken together this data support the assumption, that a single epitope peptide vaccination may result in inter-individual sharing of TCR structures [284], but also indicates that predominancy of a specific clone may be the result of individual and rather independent circumstances.

5.6 Summary and outlook

To briefly summarize our results, the generated data demonstrates that in our specific setting, in which AML patients received consecutive vaccinations with HLA-A201-restricted $WT1_{126–134}$ epitope together with GM-CSF and KLH, impaired vaccine efficacy is mainly attributed to restricted T cell functionality. As summarized in Figure 41, immune resistance mechanism exerted by leukemic blasts do not generally influence clinical outcome in our setting. Neither do inert immunoregulative mechanisms like T_{reg} or MDSC, respectively.
There is evidence that these mechanisms may have effects in individual cases, but general correlation with vaccine efficacy could not be demonstrated according to our results. In contrast, restrained vaccine efficacy seems to be a result of impaired effector phase of the immune system, as seen by an initial high level of anergic markers in patient which exhibit a quite short PFS. The failure to induce a long-term memory is mirrored by the decrease of T cells expressing the homing-receptor CCR7 as well as by the observation that WT1-specific T cells may not be able to migrate to

the site of tumor at relapse. Our results indicate that the vaccine-induced increase in $CCR7^{+}CD8^{+}$ memory T cells may represent a surrogate marker for clinical efficacy in vaccination and warrants evaluation in future clinical trials. Based on these data, future tasks concerning vaccination therapies should try to ameliorate the induction and maintenance of tumor-specific T cells. Further, the appropriate migration of these cells to the lymph nodes and SLOs, as well as to the actual tumor site should be provided by the vaccine regimen.

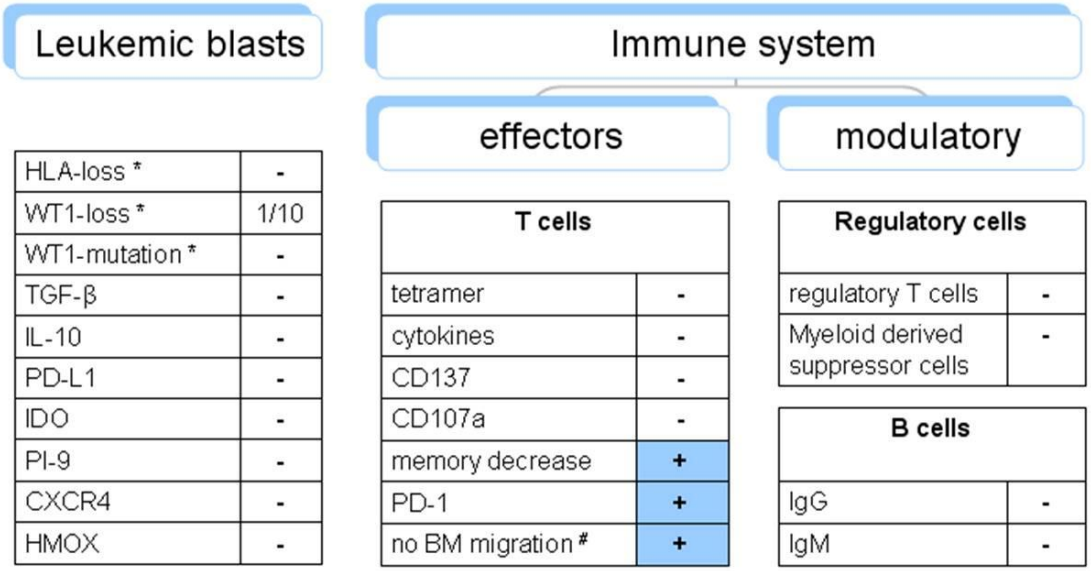


Figure 41: Mechanisms of immune resistance during vaccination: correlation with unfavorable clinical course, progression-free survival or relapse. * Data generated by Busse *et al.* [128]; # Data generated by Ochsenreither *et al.* [284]

Additionally, our data suggest that the PD-1/PD-L1 pathway plays an important role in limiting the host immune response in AML patients vaccinated with WT1 peptide, KLH and GM-CSF. On this account, and as recently proposed by others [16, 289], the evaluation of protective blocking antibodies against PD-L1 or PD-1 in patients with leukemia should be strongly considered (*cf.* Figure 11, section 1.5.2). This issue would be of special concern in vaccination therapy, as consecutive vaccination leads to chronic activation of anti-tumor T cells, one major cause for PD-1 induction.

A special point, in relation to peptide-based vaccinations is the predominant administration of single epitopes to elicit a tumor-specific immune response. As seen in our trial, the application of a specific epitope restricted to MHC-class I molecules does not sufficiently activate the entire immune repertoire. The administration of the adjuvant KLH indeed mimics the co-stimulation of $CD4^{+}$ T cells by an otherwise

presented epitope via MHC-class II, but it seems that for an effective tumor response the identical provenance of both stimuli is beneficial. For this reason, future vaccination designs should either shift to the administration of combined MHC-class I and MHC-class II restricted epitopes of tumor antigens or to overlapping peptides. The latter peptides faciliate the uptake and processing by APCs and should result in a stable presentation of epitopes on both classes of MHC molecules circumventing the competitive kinetics of actual epitope based therapies.

An alternative and interesting approach, represent BiTE-(bispecific T cell-engager)-antibodies. These antibodies are constituted of two oppositely directed variable regions of conventional immunoglobulins that are connected by a linker to form a single polypeptide chain and have been designed to transiently connect any T cell with cancer cells for initiation of redirected target cell lysis [309, 310]. All BiTE antibodies share the anti-CD3 specific single-chain antibody linked to an individual target-antigen-specific single-chain antibody, which allows T cell activation and potentially directs any CTL to deal with tumor cells.

However, despite the recent promising results in the field, cancer immunotherapy still offers a lot to learn about the underlying immunological mechansisms of cancer vaccination and its improval. This work and that of others show that cancer vaccination remains a prosperous, attractive and very important field of research that actually has to deal with four main tasks: a) the adaption of a ranking process of exisiting and novel tumor antigens, b) the alignment of criteria for the immunological evaluation of cancer vaccine trials, c) the improvement of biological adjuvants to trigger specific immune responses and d) the investigation and application of bystander-therapies reducing immune evasive and/or immune modulative effects.

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Erklärung an Eides statt

Hiermit erkläre ich, gemäß der Promotionsordnung der Humboldt-Universität zu Berlin vom 01.09.2005, dass

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